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(54) Title: STIMULATION OF ANGIOGENESIS VIA SYNDECAN-4 CYTOPLASMIC DOMAIN SIGNALING PATHWAY

(57) Abstract

The present invention provides a methodology and compositions for stimulating angiogenesis in-situ within viable cells, tissues and organs comprising endothelial cells. The methodology focuses upon and controls the phosphorylation of the 183rd amino acid residue, serine within the cytoplasmic domain and intracellular tail of transmembrane syndecan-4 proteoglycans which are then positioned at and through the cellular membrane of viable endothelial cells. By intervening and maintaining the 183rd residue in a non-phosphorylated state, a consequential cascade of intracellular events is initiated which result in a stimulation of angiogenesis in-situ.

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# STIMULATION OF ANGIOGENESIS VIA SYNDECAN-4 CYTOPLASMIC DOMAIN SIGNALING PATHWAY

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## PROVISIONAL PATENT APPLICATION

The subject matter as a whole comprising the present invention was first filed with the U.S. Patent and Trademark Office as Provisional Patent Application No. 60/073,711 on February 5, 1998.

## FIELD OF THE INVENTION

The present invention is concerned generally with the stimulation of angiogenesis in living tissues and organs; and is particularly directed to the regulation of syndecan-4 cytoplasmic domain phosphorylation within endothelial cells in-situ.

## BACKGROUND OF THE INVENTION

Angiogenesis, by definition, is the formation of new capillaries and blood vessels within living tissues; and is a complex process first recognized in studies of wound healing and then within investigations of experimental tumors. Angiogenesis is thus a dynamic process which involves extracellular matrix remodeling, endothelial cell migration and proliferation, and functional maturation of endothelial cells into mature blood vessels [Brier, G. and K. Alitalo, Trends Cell Biology 6: 454-456 (1996)]. Clearly, in normal living subjects, the process of angiogenesis is a normal host response to injury; and as such, is an integral part of the host body's homeostatic mechanisms.

It will be noted and appreciated, however, that whereas angiogenesis represents an important component part of tissue response to ischemia, or tissue wounding, or tumor-initiated neovascularization, relatively little new blood vessel formation or growth takes place in most living tissues and organs in mature adults (such as the myocardium of the living heart) [Folkman, J. and Y. Shing, J. Biol. Chem. 267: 10931-10934 (1992); Folkman, J., Nat. Med. 1: 27-31 (1995); Ware, J.A. and M. Simons, Nature Med. 3: 158-164 (1997)]. Moreover, although regulation of an angiogenetic response in-vivo is a critical part of normal and pathological homeostasis, little is presently known about the control mechanisms for this process.

A number of different growth factors and growth factor receptors have been found to be involved in the process of stimulation and maintenance of angiogenetic responses. In addition, a number of extracellular matrix components and cell membrane-associated proteins are thought to be involved in the control mechanisms of angiogenesis. Such proteins include SPARC [Sage et al., J. Cell Biol. 109: 341-356 (1989); Motamed, K. and E.H. Sage, Kidney Int. 51: 1383-1387 (1997)]; thrombospondin 1 and 2 respectively [Folkman, J., Nat. Med. 1: 27-31 (1995); Kyriakides et al., J. Cell Biol. 140: 419-430 (1998)]; and integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ [Brooks et al., Science 264: 569-571 (1994); Friedlander et al., Science 270: 1500-1502 (1995)]. However, it is now recognized that a major role is played by heparan-binding growth factors such as basic fibrocyte growth factor (bFGF) and

vascular endothelial growth factor (VEGF); and thus the regulation of angiogenesis involves the extracellular heparan sulfate matrix and the core proteins at the surface of endothelial cells.

While growth factor signalling generally occurs through specific high-affinity receptors, several growth factors are now known to interact with adjacent, membrane-anchored, proteoglycan co-receptors. In particular, bFGF requires binding to a specific sequence of sulfated polysaccharides in the extracellular heparan sulfate glycosaminoglycan (GAG) chain [Turnbull et al., J. Biol. Chem. 267: 10337-10341 (1992)] in order to bind to its high-affinity receptor on the cell surface and to exert its effect on the target cells [Olwin, B.B., and A. Rapraeger, J. Cell Biol. 118: 631-639 (1992); Rapraeger et al., Science 252: 1705-1708 (1991)]. The current picture of the role of heparan sulfate in the binding mechanism of bFGF involves dimerization of the growth factor as well as direct heparan sulfate binding to the high-affinity receptor [Brickman et al., J. Biol. Chem. 270: 24941-24948 (1995); Kan et al., Science 259: 1918-1921 (1993)]. Together, these events lead to receptor multimerization and to tyrosine trans-phosphorylation of adjacent FGF receptor cytoplasmic tails, followed by phosphorylation of other downstream substrates [Krufka et al., Biochemistry 35: 11131-11141 (1996); van der Geer et al., Annu. Rev. Cell Biol. 10: 251-337 (1994)].

Research investigations have shown that heparan sulfate core proteins or proteoglycans mediate both heparin-binding growth factors and receptor interaction at the cell surface; and that accumulation and storage of such growth factors within the extracellular matrix proper typically occurs [Vlodavsky et al., Clin. Exp. Metastasis 10: 65 (1992); Olwin, B.B. and A. Rapraeger, J. Cell Biol. 118: 631-639 (1992); Rapraeger, A.C., Curr. Opin. Cell Biol. 5: 844-853 (1993)]. The presence of heparin or heparan sulfate is thus required for bFGF-dependent activation of cell growth in-vitro [Yayon et al., Cell 64: 841-848 (1991); Rapraeger et al., Science 252: 1705-1708 (1991)]; and the removal of heparan sulfate chains from the cell surface and extracellular matrix by enzymatic digestion greatly impairs bFGF activity and inhibits neovascularization in-vivo [Sasisekharan et al., Proc. Natl. Acad. Sci. USA 91: 1524-1528 (1994)]. Ample scientific

evidence now exists which demonstrates that any meaningful alteration of heparan sulfate (HS) chain composition on the cell surface or within the extracellular matrix (which can be initiated by means of an altered synthesis, or a degradation, or a substantive modification of glycosaminoglycan chains) can meaningful affect the intracellular signaling cascade initiated by the growth factor. The importance of heparan sulfate in growth factor-dependent signaling has become well recognized in this field.

Heparan sulfate (HS) chains on the cell surface and within the extracellular matrix are present via a binding to a specific category of proteins commonly referred to as "proteoglycans". This category is constituted of several classes of core proteins, each of which serve as acceptors for a different type of glycosaminoglycan (GAG) chains. The GAGs are linear co-polymers of N-acetyl-D-glycosamine [binding heparan sulfate] or N-acetyl-D-galactosamine [binding chondroitin sulfate (CS) chains] and aoidic sugars which are attached to these core proteins via a linking tetrasaccharide moiety.

Three major classes of HS-carrying core proteins are present in living endothelial cells: cell membrane-spanning syndecans, GPI-linked glypicans, and a secreted perlecan core protein [Rosenberg et al., J. Clin. Invest. 99: 2062-2070 (1997)]. While the perlecan and glypican classes carry and bear HS chains almost exclusively, the syndecan core proteins are capable of carrying both HS and CS chains extracellularly. The appearance of specific glycosaminoglycan chains (such as HS and/or CS) extracellularly on protein cores is regulated both by the structure of the particular core protein as well as via the function of the Golgi apparatus intracellularly in a cell-type specific manner [Shworak et al., J. Biol. Chem. 269: 21204-21214 (1994)].

Today, it is recognized that the syndecan class is composed of four closely related family proteins (syndecan-1,-2,-3 and -4 respectively) coded for by four different genes in-vivo. Syndecans-1 and -4 are the most widely studied members of this class and show expression in a variety of different cell types including epithelial, endothelial, and vascular smooth muscle cells, although expression in quiescent tissues is at a fairly low level [Bernfield et al., Annu. Rev. Cell Biol. 8: 365-393 (1992); Kim et al., Mol. Biol. Cell 5: 797-805 (1994)]. Syndecan-2 (also

known as fibroglycan) is expressed at high levels in cultured lung and skin fibroblasts, although immunocytochemically this core protein is barely detectable in most adult tissues. However, syndecan-3 (also known as N-syndecan) demonstrates a much more limited pattern of expression, being largely restricted to peripheral nerves and central nervous system tissues (although high levels of expression are shown in the neonatal heart) [Carey et al., J. Cell Biol. 117: 191-201 (1992)]. All four members of the syndecan class are capable of carrying both HS and CS chains extracellularly, although most of syndecan-associated biological effects (including regulation of blood coagulation, cell adhesion, and signal transduction) are largely thought to be due to the presence of HS chains capable of binding growth factors, or cell adhesion receptors and other biologically active molecules [Rosenberg et al., J. Clin. Invest. 99: 2062-2070 (1997)].

Syndecan-1 expression has been also observed during development suggesting a potential role in the epithelial organization of the embryonic ectoderm and in differential axial patterning of the embryonic mesoderm, as well as in cell differentiation [Sutherland et al., Development 113: 339-351 (1991); Trautman et al., Development 111: 213-220 (1991)]. Also, mesenchymal cell growth during tooth organogenesis is associated with transient induction of syndecan-1 gene expression [Vainio et al., Dev. Biol. 147: 322-333 (1991)]. Furthermore, in adult living tissues, expression of syndecan-1 and syndecan-4 proteoglycans substantially increases within arterial smooth muscle cells after balloon catheter injury [Nikkari et al., Am. J. Pathol. 144: 1348-1356 (1994)]; in healing skin wounds [Gallo et al., Proc. Natl. Acad. Sci. USA 91: 11035-11039 (1994)]; and in the heart following myocardial infarction [Li et al., Circ. Res. 81: 785-796 (1997)]. In the latter instances, the presence of blood-derived macrophages appears necessary for the induction of syndecan-1 and -4 gene expression.

Presently, however, the effects of changes in syndecan expression on cell behavior are not well understood. For example, this core protein is believed to mediate bFGF binding and cell activity. Overexpression of syndecan-1 in 3T3 cells led to inhibition of bFGF-induced growth [Mali et al., J. Biol. Chem. 268: 24215-24222 (1993)]; while in 293T cells, overexpression of syndecan-1 augmented serum-dependent growth [Numa et al., Cancer Res. 55: 4676-4680

(1995)]. Furthermore, syndecan-1 overexpression showed increased inter-cellular adhesion in lymphoid cells [Lebakken et al., J. Cell Biol. 132: 1209-1221 (1996)] while also decreasing the ability of B-lymphocytes to invade collagen gels [Libersbach, B.F. and R.D. Sanderson, J. Biol. Chem. 269: 20013-20019 (1994)]. These ostensibly contradictory findings have merely added to the uncertainty and the disparity of knowledge regarding the effects of syndecan expression.

In addition, although there are significant differences between the sequences of their ectoplasmic domains, the four syndecans share a highly conserved cytoplasmic tail containing four invariant tyrosines and one invariant serine [Kojima et al., J. Biol. Chem. 267: 4870-4877 (1992)]. This degree of conservation may reflect functional similarities between cytoplasmic tails of all the syndecans. However, unlike the well established involvement of the ectoplasmic domain in growth factor binding through the GAG chains, there is still no consensus regarding the function of the cytoplasmic tail. Several reports [Carey et al., J. Cell Biol. 124: 161-170 (1994); Carey et al., Exp. Cell Res. 214: 12-21 (1994)] point to transient association of the cytoplasmic tail of syndecan-1 to the actin cytoskeleton which seems to be highly dependent on the presence of one of the four conserved tyrosines [Carey et al., J. Biol. Chem. 271: 15253-15260 (1996)].

It is recognized also that the 18-amino acid-long cytoplasmic tail of syndecan-4 is the least homologous to the other three syndecans, containing a unique nine-residue sequence (RMKKKDEGSYDLGKKPIYKKAPTNEFYA). Syndecan-4 is incorporated into focal adhesions of fibroblasts in a PKC-dependent manner [Baciu, P.C. and P.F. Goetinck, Mol. Biol. Cell 6: 1503-1513 (1995)]; and its cytoplasmic tail appears to bind and activate PKCα [Oh et al., J. Biol. Chem. 272: 8133-8136 (1997)]. These capacities are special to the cytoplasmic tail of syndecan-4 and not shared by the other syndecans, because they are mediated through oligomerization of its unique nine-residue sequence [Oh et al., J. Biol. Chem. 272: 11805-11811 (1997)].

Also, the presence of the five conserved phosphorylatable residues in the cytoplasmic tails of all the syndecans has been noted. However, although in-vitro phosphorylation by calcium-dependent PKC of serine residues in partial or

complete synthetic cytoplasmic tails was reported for syndecan-2 and syndecan-3, it could not be produced for syndecan-1 or syndecan-4 [Prasthofer et al., Biochem. Mol. Biol. Int. 36: 793-802 (1995); Oh et al., Arch. Biochem. Bio Phys. 344: 67-74 (1997)]. Serine phosphorylation in situ was detected in syndecan-2 of carcinoma cells cultured in the presence of serum [Itano et al., Biochem. J. 325: 925-930 (1996)]. This phosphorylation was attributed to the serine residue in the cytoplasmic tail of syndecan-2, contained within a sequence that conforms to a phosphorylation motif of cAMP and cGMP-dependent kinases. In situ phosphorylation of the cytoplasmic tail of syndecan-1 was produced in mammary gland cells by treatment with orthoyanadate or pervanadate, both of which inhibit tyrosine phosphatase [Reiland et al., Biochem. J. 319: 39-47 (1996)]. Accordingly, this treatment resulted predominantly in tyrosine phosphorylation, although a lesser degree of serine phosphorylation was also detected. One of the four tyrosines in the cytoplasmic tail of syndecan-1 is contained within a tyrosine kinase phosphorylation motif [Gould et al., Proc. Natl. Acad. Sci. USA 89: 3271-3275 (1992)] conserved in all the syndecans and may at least partially account for the orthovanadate and pervanadate-produced phosphorylation.

In sum therefore, it is evident that the quantity and quality of knowledge presently available regarding glycoseaminoglycan (GAG) binding core proteins is factually incomplete, often presumptive, and in some instance apparently contradictory. Clearly the role of specific proteoglycans, and particularly syndecans, as mediators under various conditions is recognized; nevertheless, the mechanisms of action and the functional activity of the various individual syndecan core proteins remains yet to be elucidated. Thus, while the role of proteoglycans generally is known to relate in some manner to angiogenesis, there is no evidence or data as yet which establishes the true functional action of specific proteoglycans nor which provides a means for using specific proteoglycans to stimulate angiogenesis in-situ.

## SUMMARY OF THE INVENTION

The present invention is comprised of related alternatives and has multiple aspects. One aspect provides a first method for stimulating angiogenesis within various tissues and organs in-situ, said method comprising:

identifying a viable endothelial cell in-situ as a target, said targeted endothelial cell bearing a plurality of transmembrane syndecan-4 proteoglycans positioned at and through the cell surface wherein the 183rd amino acid residue present within the intracellular cytoplasmic domain of said syndecan-4 proteoglycan is a serine residue;

administering to said targeted endothelial cell on at least one occasion a predetermined amount of an inhibitor of Protein Kinase C δ (delta) isoenzyme activity such that said 183rd serine residue within the cytoplasmic domain of at least some of said syndecan-4 proteoglycans is present in a non-phosphorylated state; and

allowing said 183rd serine residue within the cytoplasmic domain of said syndecan-4 proteoglycans to continue to be present in a non-phosphorylated state, whereby a stimulation of angiogenesis in-situ results.

Another aspect provides a related, but alternative method for stimulating angiogenesis within viable cells, tissues, and organs in-situ, said alternative method comprising:

identifying a viable endothelial cell in-situ as a target, said targeted endothelial cell bearing a plurality of transmembrane syndecan-4 proteoglycans positioned at and through the cell surface wherein the 183rd amino acid residue present within the intracellular cytoplasmic domain of said syndecan-4 proteoglycan is a serine residue;

administering to said targeted endothelial cell on at least one occasion a predetermined amount of a composition able to increase Protein Kinase C α (alpha) isoenzyme activity intracellularly such that said 183rd serine residue within the cytoplasmic domain of at least some of said syndecan-4 proteoglycans is present in an non-phosphorylated state in-situ; and

allowing said 183rd serine residue within the cytoplasmic domain of said syndecan-4 proteoglycans to continue to be present in a non-phosphorylated state, whereby a stimulation of angiogenesis in-situ results.

A different aspects provides another related, but alternative method for stimulating angiogenesis within viable cells, tissues, and organs in-situ, said alternative method comprising:

identifying a viable endothelial cell in-situ as a target, said targeted endothelial cell bearing a plurality of transmembrane syndecan-4 proteoglycans positioned at and through the cell surface wherein the 183rd amino acid residue present within the intracellular cytoplasmic domain of said syndecan-4 proteoglycan is a serine residue;

administering to said targeted endothelial cell on at least one occasion a predetermined amount of an substance able to activate at least one enzyme selected from the group consisting of protein phosphatases 1 and 2A such that said 183rd serine residue within the cytoplasmic domain of at least some of said syndecan-4 proteoglycans is present in an non-phosphorylated state; and

allowing said 183rd serine residue within the cytoplasmic domain of said syndecan-4 proteoglycans to continue to be present in a non-phosphorylated state, whereby a stimulation of angiogenesis in-situ results.

## BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more easily understood and better appreciated when taken in conjunction with the accompanying drawing, in which:

Figs. 1A and 1B are photographs showing the detection of syndecan-4 core protein basal phosphorylation and identification of serine phosphorylation;

Figs. 2A and 2B are photographs showing the localization of syndecan-4 core protein phosphorylation to the cytoplasmic tail;

Figs. 3A and 3B are graphs showing the effects of bFGF and calyculin on syndecan-4 cytoplasmic tail phosphorylation;

Figs. 4A and 4B are photographs and a graph showing the effects of PKC activation and inhibition on syndecan-4 cytoplasmic tail phosphorylation;

Fig. 5 is a graph with insert showing the activation of PKC α (alpha) isoenzyme by syndecan-4 cytoplasmic tail peptides;

Figs. 6A-6C are photographs showing PKC binding to syndecan-4 cytoplasmic tail peptides;

Figs. 7A and 7B are photographs and a graph showing the binding between $PIP_2$ and syndecan-4 cytoplasmic tail peptides;

Figs. 8A-8D are graphs showing the results of size-exclusion column chromatography of syndecan-4 cytoplasmic tail peptides.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides both tangible means and methods for causing the 183rd residue, serine, in the cytoplasmic domain of syndecan-4 core proteins of vascular endothelial cells to exist in a non-phosphorylated state in-situ; and by this intervention and meaningful change, consequentially to effect a stimulation of angiogenesis at the local anatomic site in-vivo.

A number of major benefits and advantages are therefore provided by the means and methods comprising the present invention. These include the following:

1. The present invention provides in-situ stimulation for angiogenesis. By definition, therefore, both in-vivo and in-vitro circumstances of use and application are envisioned and expected. Moreover, the vascular endothelial cells which are suitable for treatment using the present methods may alternatively include and be isolated endothelial cells, part of living tissues comprising a variety of other cells such as fibroblasts and muscle cells, and also comprise part of specific organs in the body of a living human or animal subject. While the user shall choose the specific conditions and circumstances for practicing the present invention, the intended scope of application and the envisioned utility of the means and methods described herein apply broadly to living cells, living tissues, functional organs and systems, as well as the complete living body unit as a viable whole.

2. The present invention has a variety of different applications and uses. Of clinical and medical interest and value, the present invention provides the opportunity to stimulate angiogenesis in tissues and organs in a living subject which has suffered defects or has undergone anoxia or infarction. A common clinical instance is the myocardial infarction or chronic myocardial ischemia of heart tissue in various zones or areas of a living human subject. The present invention thus provides opportunity and means for specific site stimulation and inducement of angiogenesis under controlled conditions. The present invention also has major research value for research investigators in furthering the quality and quantity of knowledge regarding the mechanisms controlling angiogenesis under a variety of different conditions and circumstances.

3. The present invention envisions and permits a diverse range of routes of administration and delivery means for introducing a variety of synthetically constructed oligonucleotide expression vectors to a specific location, site, tissue, organ, or system in the living body. A variety of different vectors are available to the practitioner; and a diverse and useful range of delivery systems which are conventionally available and in accordance with good medical practice are adapted directly for use. In this manner, not only are the means for stimulating angiogenesis under the control of the user, but also the manner of application and the means for limiting the locale or area of affected vascular endothelial cells can be chosen and controlled.

4. The present invention provides a unique capability and control for stimulating angiogenesis in-situ by genetic manipulation of the endothelial cells as they exist within the tissues and organs as found. This level of control and utilization of the mechanisms found within the cytoplasms of the endothelial cells themselves provides a point of intentional intervention which harnesses and utilizes the cellular systems of the endothelial cells themselves to produce the intended and desired result. The affected endothelial cells in-situ are thus minimally altered; and the methodology utilizes the natural regulatory and protein producing systems of the endothelial cells themselves to provide the desired effect upon syndecan-4 proteoglycans which are located and positioned normally by the endothelial cells as part of the normal homeostatic mechanisms.

## I. Underlying Basis Of The Invention

Recent research investigations have shown that a member of the syndecan family of heparan sulfate-carrying proteoglycans participates in intracellular signalling via its cytoplasmic tail. This particular transmembrane proteoglycan, syndecan-4, is a ubiquitous molecule present in-vivo within most human cells and tissues, including the vascular endothelium. The present invention utilizes and takes advantage of syndecan-4's cellular function in order to control and upregulate new blood vessel growth and to promote angiogenesis, particularly in the heart.

The underlying premise of the present invention is that phosphorylation of the cytoplasmic tail of syndecan-4 at the $Ser^{183}$ residue regulates a member of the protein kinase C enzyme family (PKCα), whose specific enzymatic activity is essential for proliferation and migration of endothelial cells in-vivo. As empirically demonstrated, the $Ser^{183}$-dephosphorylated residue in the syndecan-4 molecule can significantly increase the catalytic activity of PKCα, while the phosphorylated $Ser^{183}$ residue form of syndecan-4 merely activates PKCα. This result and effect is empirically proven by the experiments and data presented hereinafter. Thus, by inhibiting the Protein Kinase C isoenzyme responsible for $Ser^{183}$ phosphorylation, and/or by activating the corresponding phosphatase that removes the phosphate group from the $Ser^{183}$ residue, the means now exist to increase PKCα activity, and to promote endothelial cell proliferation and migration in-situ.

Several lines of evidence further reveal the relation between the phosphorylation level of the syndecan-4 cytoplasmic domain and its associated effects on PKCα, and the consequential proliferation and migration activities of endothelial cells. First and foremost, a significant decrease in syndecan-4 phosphorylation is observed upon cell treatment with bFGF. Thus, the occurrence of syndecan-4 dephosphorylation in the cytoplasmic tail region accompanies the proliferative response of the endothelial cells to one of the most ubiquitous growth factors, and one which is secreted in response to clinical ischemia and infarction. Second, it has only recently been observed that dephosphorylation of the cytoplasmic tail of syndecan-4 is required for its activation of PKCα. Thus,

syndecan-4 phosphorylation is an event directly linked to a PKC isoenzyme which is known to promote endothelial cells migration. Third, empirical data indicate that endothelial cells, in which PKCα has been suppressed by transfection with an inactive form of this enzyme kinase, proliferate at a much slower rate than wild type cells which have not been suppressed. Fourth, and finally, endothelial cells transfected with a syndecan-4 mutant bearing a $Ser^{183}$-$Glu^{183}$ replacement (which mimics the conferral of a negative charge by phosphorylation) also proliferated at a lower rate than wild type endothelial cells. Taken together, all these findings provide confirmation and evidentiary support for the role of syndecan-4 cytoplasmic tail phosphorylation in endothelial cell migration and proliferation. Consequently, control and regulation of syndecan-4 cytoplasmic tail phosphorylation is a potent methodology suitable and effective as therapies aimed at promoting angiogenesis.

## II. The Syndecan-4 Cytoplasmic Domain

It will be recalled that the 28 amino acid-long cytoplasmic tail of syndecan-4 is the least homologous to the other three syndecans, containing a unique nine amino acid residue sequence (shown in bold type)

RMKKKDEGSYDL**GKKPIYKK**APTNEFYA

Syndecan-4 is known to be incorporated into focal adhesions of fibroblasts in a PKC-dependent manner and its cytoplasmic tail in the phosphorylated state binds and activates PKCα directly. These capacities and functions are special to the cytoplasmic tail of syndecan-4; and these capacities and functions are not shared by the other syndecans, since they are mediated through oligomerization of the syndecan-4 cytoplasmic tail's unique nine-residue sequence.

The critical and essential target of the present methods, therefore, is the serine residue located in-situ as the 183rd amino acid in the syndecan-4 molecular structure and existing in proximity to the unique nine amino acid residue sequence of the 28 residue-long cytoplasmic tail. This individual serine residue is the sole

and exclusive site of interest; and it appears that no other individual amino acid residue and no peptide segment within the cytoplasmic domain is involved in any major degree.

Equally important, it will be recognized and appreciated that it is the state of this serine$^{183}$ residue - as being either non-phosphorylated or phosphorylated - which provides the invention with the means for and the effect of regulatory control. Thus, the larger the number of syndecan-4 core proteins whose intracellular cytoplasmic domain comprises an unphosphorylated serine$^{183}$ residue, the greater the upregulation of PKCα isoenzyme catalysis and the greater the inducement of angiogenesis in-situ. Conversely, the larger the number of syndecan-4 proteoglycans having a phosphorylated serine$^{183}$ residue as part of their intracellular cytoplasmic domains, the smaller the degree of PKCα isoenzyme catalysis and the more limited the amount of angiogenesis in-situ.

## III. The Methodology Comprising The Present Invention

The goal and objective of the present invention is to prevent the phosphorylation of or to decrease the phosphorylation level of Ser$^{183}$ residue in the cytoplasmic domain or tail of syndecan-4 molecules then present and existing within vascular endothelial cells. The phosphorylation level of the Ser$^{183}$ residue is normally an outcome of a dynamic equilibrium between the catalytic activities of a pair of enzymes - of a specific protein kinase and a phosphatase that incorporate or remove, respectively, a phosphate group. These two enzymes are, therefore, the individual objects to be manipulated in a variety of modes. The categorical methods and goals of these manipulations thus are and include:

(A) Means and procedures to inhibit the protein kinase responsible for the phosphorylation of Ser$^{183}$ in the cytoplasmic tail of syndecan-4. This kinase has been identified and empirically shown to be the calcium-independent PKCδ (delta) isoenzyme.

(B) Means and procedures to increase PKCα (alpha) activity. The dephosphorylated cytoplasmic tail of syndecan-4 has been empirically shown to promote PKCα (alpha) activity, which directly increases the angiogenic potential of endothelial cells in-situ.

(C) Means and procedures to activate the phosphatase responsible for the dephosphorylation of the $Ser^{183}$ residue in-situ. Though the specific phosphatase has not been completely identified as yet, it belongs to and is part of the protein phosphatase type 1/2A family.

Composition Preparation And Manufacture

As a point of information also, it will be recognized and appreciated that in terms of preparing and using suitable inhibitory agents and compositions, it is important, if not essential, that the user be at least familiar with the many established procedures and conventionally known techniques for manipulating and modifying nucleotides and DNA (and RNA) fragments as well as the vectors to carry them which have been reported and are today widespread in use and application. Merely exemplifying the many authoritative texts and published articles presently available in the literature regarding genes, DNA nucleotide manipulation and the expression of proteins from manipulated DNA fragments are the following: Gene Probes for Bacteria (Macario and De Marcario, editors) Academic Press Inc., 1990; Genetic Analysis, Principles Scope and Objectives by John R.S. Ficham, Blackwell Science Ltd., 1994; Recombinant DNA Methodology II (Ray Wu, editor), Academic Press, 1995; Molecular Cloning, A Laboratory Manual (Maniatis, Fritsch, and Sambrook, editors), Cold Spring Harbor Laboratory, 1982; PCR (Polymerase Chain Reaction), (Newton and Graham, editors), Bios Scientific Publishers, 1994; and the many references individually cited within each of these publications. All of these published texts are expressly incorporated by reference herein.

## A. Inhibition of PKCδ (delta) Isoenzyme Activity

(A.1) Chemical PKC Inhibitory compounds:

A vast array of chemical PKC inhibitors has been developed and is commercially available. Examplifying such inhibitors is staurosporine, a substance obtained from *Streptomyces* species. Others are listed below in Table 1. However, most of these inhibitors are non-selective and equally potent against all types of PKC isoenzymes. One noted exception of the calcium-dependent PKC isoenzyme inhibitor is Gö 6976 [Martiny-Baron et al., J. Biol. Chem. 268: 9194-9197 (1993)]. In so far as is presently known, however, there is no specific chemical inhibitor of the calcium-independent PKC isoenzymes in general, or of the PKCδ (delta) isoenzyme itself. For these reasons, a more specific inhibition of PKCδ alone is desirable and can be achieved by using one of the alternatives given below.

Table 1: Representative PKC Inhibitors

| Inhibitor | $IC_{50}$ (in $\mu M$) |
|---|---|
| Calphostin C | 0.05 |
| Chelerythmine chloride | 0.66 |
| Gö 6976 | 0.008 |
| Autoinhibitory peptide | 15 |
| Staurosporine | 0.0007 |

(A.2) Overexpression of the autoinhibitory domain of PKCδ (delta) isoenzyme:

The regulatory domain of all PKC isoenzymes contains a sequence motif similar to the consensus sequence found in most PKC substrates in which the Thr or Ser residue normally phosphorylated by PKC is replaced by an Ala residue. In the inactive state of the enzyme, this motif blocks the catalytic domain and prevents enzyme interaction with its potential substrates. Induced over-expression of the pseudosubstrate domain of PKCδ will therefore reduce the activity of this isoenzyme by competing with its cellular substrates. Moreover, the pseudosubstrate domain of PKCδ is sufficiently different from those of the other PKC isoenzymes so as not to interfere with their catalytic activity. The pseudosubstrate domain of PKCδ (delta) isoenzyme is provided by Table 2 below.

It will be recognized that the information of Table 2 is a reproduction in part from Nishikawa et al., J. Biol. Chem. 272: 952-960 (1997), the full text of which is expressly incorporated by reference herein.

Table 2: The Pseudosubstrate Domain of the PKCδ (delta) Isoenzyme

AARKRKGSFFYGG

Over-expression of the PKCδ pseudosubstrate can be produced within the endothelial cell at the target region by transfection with the adenovirus containing a cDNA construct with the PKCδ pseudosubstrate sequence of Table 2. An established protocol and procedural detail has been reported and published in the scientific literature by Nishikawa et al., J. Biol. Chem. 272: 952-960 (1997); the text of this publication and its cited references is expressly incorporated by reference herein. The prepared adenovirus vector carrying the inserted DNA coding for the pseudosubstrate domain of the PKCδ (delta) isoenzyme can be administered by direct injection or infusion to the local anatomic site of the host in-vivo over a schedule dose administration over a period of days.

(A.3) Expression of PKCδ anti-sense constructs

Transfection with the anti-sense cDNA of a protein is a frequently used method for suppressing the expression of that protein. The mRNA transcribed from the transfected cDNA shuts off the translation of the target protein by hybridizing to the sense endogenous mRNA message. The delivery method of the anti-sense cDNA is similar to the one used above for over-expressing the pseudosubstrate domain of PKCδ, preferably also using the adenovirus vector for administration.

As a representative specific example of an antisense entity useful with the present invention, the PKCδ (delta) antisense sequences (I) and (II) are given below.

(I) PKC Delta antisense primer (42 mer):
5'-GGC CGC TGG GCA TCG AAC GTC GAC TTC CAC TCA GGA TAC ATG-3'

(II) PKC Delta antisense complement primer (42 mer):
5'-GAT CCA TGT ATC CTG AGT GGA AGT CGA CGT TCG ATG CCC AGC-3'

It will be recognized and appreciated that these two embodiments are merely representative and illustrative of this type of inhibitory compound; and that a wide range of other DNA oligonucleotide fragments can be prepared which will be useful and functional in varying degrees of efficacy.

(A.4) Delivery of anti-sense oligodeoxynucleotides (ODN)

In this approach, a short (around 15-30 base pairs) ODN with an anti-sense sequence corresponding to a chosen sequence in the cDNA of the protein intended for suppression is introduced in the target cells. The principle of this method is similar to that of the anti-sense construct, but instead of interfering with translation of the mRNA, this approach interferes with transcription by hybridization between of exogenous anti-sense ODN to the corresponding locus in the genomic DNA.

## B. Increases in PKCα (alpha) Isoenzyme Activity

(B.1) Chemically Induced Increases in PKCα Activity

In addition to enhancing PKCα activity through prevention of syndecan-4 cytoplasmic tail phosphorylation, this PKCα activity can be increased directly. The commonly used compounds for increasing PKC activity are phorbol esters, but these compositions are not isoenzyme-specific. A representative listing of useful phorbol esters and other PKC activators, all of which are commercially available, is provided by Table 3 below. In addition, however, an increase in PKC activity limited to the α (alpha) isoenzyme can be achieved using one of the alternative methods described below.

Table 3: Representative Phorbol Ester Compositions

ADMB;

Ingenol;

1,2-Didecanoyl-rac-glycerol-Mezerein;

Phorbol-12-myristate-13-acetate (PMA);

1-stearoyl-2-arachidonoyl-SN-glycerol;

12-0-tetradecanoyl-phorbol-13-acetate (TPA)

(B.2) Over-expression of full-length PKCα molecules

A marked increase in the number and abundance of PKCα (alpha) isoenzyme molecules will elevate the phosphorylation level of its downstream substrates, resulting in higher proliferative and migratory cellular activities. Such increase can be achieved by introducing exogenous cDNA encoding the full-length PKCα (alpha) isoenzyme using adenoviral transfection as conventionally known and practiced. For ease of understanding and completeness of description, a recitation of cDNA encoding the entirety of the PKCα (alpha) isoenzyme is provided by Table 4 below.

In addition, the cDNA recitation of Table 4 will be recognized as a reproduction in-part of the scientific information published in Parker et al., Science 233: 853-859 (1986), the full text of which is expressly incorporated by reference herein.

Table 4: cDNA Encoding the PKCα (alpha) Isoenzyme

```
LOCUS       BOVPKIC      2324 bp    mRNA            MAM       08-APR-1987
DEFINITION  Bovine protein kinase C mRNA, complete cds.
ACCESSION   M13973
NID         g163529
KEYWORDS    kinase; phorbol ester receptor; protein kinase; protein kinase C;
            serine kinase; threonine kinase.
SOURCE      Bovine (calf) brain, cDNA to mRNA, clones lambda-bPKC(21,306).
  ORGANISM  Bos taurus
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Artiodactyla; Ruminantia; Pecora; Bovoidea;
            Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 2324)
  AUTHORS   Parker,P.J., Coussens,L., Totty,N.F., Rhee,L., Young,S., Chen,E.,
            Stabel,S., Waterfield,M.D. and Ullrich,A.
  TITLE     The complete primary structure of protein kinase C-the major
            phorbol ester receptor
  JOURNAL   Science 233, 853-859 (1986)
  MEDLINE   86269429
FEATURES             Location/Qualifiers
     source          1..2324
                     /organism="Bos taurus"
                     /db_xref="taxon:9913"
     mRNA            <1..>2324
                     /note="pkC mRNA"
     CDS             51..2069
                     /note="protein kinase C"
                     /codon_start=1
                     /db_xref="PID:g163530"
                     /translation="MADVFPAAEPAAPQDVANRFARKGALRQKNVHEVKNHRFIARFF
                     KQPTFCSHCTDFIWGFGKQGFQCQVCCFVVHKRCHEFVTFSCPGADKGPDTDDPRSKH
                     KFKIHTYGSPTFCDHCGSLLYGLIHQGMKCDTCDMNVHKQCVINVPSLCGMDHTEKRG
                     RIYLKAEVTDEKLHVTVRDAKNLIPMDPNGLSDPYVKLKLIPDPKNESKQKTKTIRST
                     LNPRWDESFTFKLKPSDKDRRLSEEIWDWDRTTRNDFMGSLSFGVSELMKMPASGWYK
catalytic domain     LLNQEEGEYYNVPIPEGDEEGNVELRQKFEKAKLGTAGNKVISPSEDRRQPSNNLDRV
(underlined)         KLTDFNFLMVLGKGSFGKVMLADRKGTEELYAIKILKKDVVIQDDDVECTMVEKRVLA
                     LLDKPPFLTQLHSCFQTVDRLYFVMEYVNGGDLMYHIQQVGKFKEPQAVFYAAEISIG
                     LFFLHKRGIIYRDLKLDNVMLDSEGHIKIADFGMCKEHMMDGVTTRTFCGTPDYIAPE
                     IIAYQPYGKSVDWWAYGVLLYEMLAGQPPFDGEDEDELFQSIMEHNVSYPKSLSKEAV
                     SICKGLMTKHPGKRLGCGPEGERDVREHAFFRRIDWEKLENREIQPPFKPKVCGKGAE
                     NFDKFFTRGQPVLTPPDQLVIANIDQSDFEGFSYVNPQFVHPILQSAV"
BASE COUNT      527 a    683 c    695 g    419 t
ORIGIN      201 bp upstream of PstI site.
        1 ccctctcggc cgccgcccgc gccccccgcg gcaggaggcg gcgagggacc atggctgacg
       61 tcttcccggc cgccgagccg gcggcgccgc aggacgtggc caaccgcttc gcccgcaaag
      121 gggcgctgag gcagaagaac gtgcacgagg tgaagaacca ccgcttcatc gcgcgcttct
      181 tcaagcagcc caccttctgc agccactgca ccgacttcat ctgggggttt gggaaacaag
      241 gcttccagtg ccaagtttgc tgttttgtgg ttcacaagag gtgccatgaa tttgttactt
      301 tttcttgtcc gggggcggat aaaggacccg acacagatga cccgaggagc aagcacaagt
      361 tcaagatcca cacgtatggc agccccacct tctgtgatca ctgcggctcc ctgctctacg
      421 gactcatcca ccagggatg aaatgtgaca cctgtgatat gaacgtgcac aagcagtgcg
      481 tgatcaacgt gcccagcctc tgcgggatgg accacacgga gaagaggggc cgcatctacc
      541 tgaaggccga ggtcacggat gaaaagctgc acgtcacagt acgagacgcg aaaaacctaa
      601 tccctatgga tccaaatggg ctttcagatc cttacgtgaa gctgaagctt attcctgacc
      661 ccaagaacga gagcaaacag aaaaccaaga ccatccgctc gacgctgaac ccccggtggg
      721 acgagtcctt cacgttcaaa ttaaaacctt ctgataaaga ccggcgactg tccgaggaaa
      781 tctgggactg ggatcgaacc acacggaacg acttcatggg gtccctttcc tttggggtct
      841 cggagctgat gaagatgccg gccagcggat ggtacaagct gctgaaccaa gaggagggcg
      901 agtactacaa cgtgccgatc cccgaaggcg acgaggaagg caatgtggag ctcaggcaga
```

Table 4: cDNA Encoding the PKCα (alpha) Isoenzyme (continued)

```
 961 aattcgagaa agccaagctt ggccctgccg gcaacaaagt catcagtccc tccgaggaca
1021 ggagacagcc ttccaacaac ctggacagag tgaagctcac ggacttcaac ttcctcatgg
1081 tgctgggcaa aggcagcttt gggaaggtga tgctggccga ccggaagggg acagaggagc
1141 tgtacgccat caagatcctg aagaaggacg tggtcatcca ggacgacgac gtggagtgca
1201 ccatggtgga gaagcgggtc ctggcgctgc tcgacaagcc gccgttcctg acgcagctgc
1261 actcctgctt ccagacggtg gaccggctgt acttcgtcat ggagtacgtc aacggcgggg
1321 acctcatgta ccacatccag caggtcggga agttcaagga gccgcaagca gtgttctatg
1381 cagcagagat ttccatcggg ctgttctttc ttcataaaag aggaatcatt tatcgggacc
1441 tgaagttaga caacgtcatg ctggactcgg aaggacacat taagatcgcg gacttcggga
1501 tgtgcaagga gcacatgatg gacggcgtca cgaccaggac cttctgcggg acccccgact
1561 acatcgcccc agagataatc gcctatcagc cgtacgggaa gtccgtggac tggtgggcct
1621 acggcgtcct gttgtacgag atgttggccg ggcagcctcc gttcgacggc gaggacgagg
1681 acgagctgtt ccagtccatc atggagcaca acgtctcgta ccccaagtcc ttgtccaagg
1741 aggccgtgtc catctgcaaa gggctgatga ccaagcaccc cgggaagcgg ctgggctgcg
1801 ggcccgaggg cgagcgcgac gtgcgggagc atgccttctt ccggaggatc gactgggaga
1861 agctggagaa ccgtgagatc cagccacccc tcaagcccaa agtgtgcggc aaaggagcag
1921 agaactttga caagttcttc acgcgagggc agcctgtctt gacgccgccc gaccagctgg
1981 tcatcgctaa catcgaccag tctgattttg aaggcttctc ctacgtcaac ccccagttcg
2041 tgcaccccat cctgcagagc gcggtatgag acgcctcgcg gaagcctggt ccgcgccccc
2101 gccccgcct ccgccccgc cgtgggaagc gacccccacc ctagggtttg ccggcctcgg
2161 ccctccctgt tccaggtgga ggcctgaaaa ctgtagggtg gttgtccccg cgtgctcggc
2221 tgcgtcatct cagcggaaga tgacgtcacg tcggcatctg cttgacgtag aggtgacatc
2281 tggcgggggа ttgacccttt ctggaaagca aacagactct ggcc
```

(B.3) Over-expression of PKCα catalytic domain

A larger increase in PKCα activity can be achieved by over-expressing only the cDNA encoding its unregulated catalytic subunit (PKM), instead of the full-length protein. The catalytic subunit (PKM) is constitutively active in and of itself; and will increase the basal activity of PKCα when introduced to a viable cell even in the absence of external activation. A recitation of the cDNA encoding the catalytic subunit (PKM) alone of the PKCα (alpha) isoenzyme is provided by Table 5 below. It will be appreciated that the recitation of Table 5 is a reproduction in part of the information presented by Parker et al., Science 233: 853-859 (1986), the full text of which is expressly incorporated by reference herein.

Table 5: cDNA Encoding the Catalytic Subunit (PKM) of PKCα (alpha) Isoenzyme

acggacttca acttcctcat ggtgctgggc aaaggcagct ttgggaaggt gatgctggcc
gaccggaagg ggacagagga gctgtacgcc attaagatcc tgaagaagga cgtggtcatc
caggacgacg acgtggagtg caccatggtg gagaagcggg tcctggcgct gctcgacaag
ccgccgttcc tgacgcagct gcactcctgc ttccagacgg tggaccggct gtacttcgtc
atggagtacg tcaacggcgg ggacctcatg taccacatcc agcaggtcgg gaagttcaag
gagccgcaag cagtgttcta tgcagcagag atttccatcg ggctgttctt tcttcataaa
agaggaatca tttatcggga cctgaagtta gacaacgtca tgctggactc ggaaggacac
attaagatcg cggacttcgg gatgtgcaag gagcacatga tggacggcgt cacgaccagg
accttctgcg ggacccccga ctacatcgcc ccagagataa tcgcctatca gccgtacggg
aagtccgtgg actggtgggc ctacggcgtc ctgttgtacg agatgttggc cgggcagcct
ccgttcgacg gcgaggacga ggacgagctg ttccagtcca tcatggagca caacgtctcg
taccccaagt ccttgtccaa ggaggccgtg tccatctgca aagggctgat gaccaagcac
cccgggaagc ggctgggctg cgggcccgag ggcgagcgcg acgtgcggga gcatgccttc
ttccggagga tc

## C. Activation of protein phosphatase 1/2A

As noted previously herein, the field of phosphatases responsible for dephosphorylating the $Ser^{183}$ in the cytoplasmic tail of syndecan-4 in-situ has been narrowed markedly to protein phosphatase type 1 (PP1) or 2A (PP2A). This fact has been experimentally verified hereinafter.

In addition, the capacity of okadaic acid to inhibit the dephosphorylation of the cytoplasmic tail of syndecan-4 is being employed to discriminate between the two enzyme types. Since protein phosphatase PP2A is more sensitive to okadaic acid than type PP1 enzyme, these experiments will serve to discriminate between the two enzyme types. After the phosphatase type is determined and precisely identified, the following procedures can be used to increase phosphatase enzyme activity in the target cell population:

(C1) Over-expression of the catalytic subunits of the protein phosphatase

Protein phosphatase type PP1 is a multimer consisting of a catalytic subunit and one of several known inhibitory subunits [Mumby, M.C. and G. Walter, Physiol. Rev. 73: 673-699 (1993)]. In comparison, protein phosphatase type PP2A is a heterotrimer of two regulatory subunits (A and B) and a catalytic subunit (C). Once it has been determined whether type PP1 or type PP2A is responsible for dephosphorylating the cytoplasmic tail of syndecan-4, the respective catalytic subunit can be over-expressed by adenoviral transfection with the cDNA of this subunit. For informational purposes, the DNA sequence for both types PP1 and PP2A is provided by Table 6 below.

Table 6

```
LOCUS       HUMPRPHOS1   1367 bp    mRNA            PRI       22-APR-1991
DEFINITION  Human protein phosphatase-1 catalytic subunit mRNA, complete cds.
ACCESSION   M63960
NID         g190515
KEYWORDS    dephosphorylate phosphoprotein; protein phosphatase-1.
SOURCE      Human liver hepatoma Hep G2 cell line, cDNA to mRNA, clone
            PP1HEPG2-B.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1367)
  AUTHORS   Tung,L.
  JOURNAL   Unpublished (1991)
FEATURES             Location/Qualifiers
     source          1..1367
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /cell_line="Hep G2"
                     /cell_type="hepatoma"
                     /tissue_type="liver"
                     /tissue_lib="Hep G2 cDNA library"
     CDS             30..1022
                     /EC_number="3.1.3.16"
                     /note="catalytic subunit"
                     /codon_start=1
                     /product="protein phosphatase-1"
                     /db_xref="PID:g190516"
                     /translation="MSDSEKLNLDSIIGRLLEVQGSRPGKNVQLTENEIRGLCLKSRE
                     IFLSQPILLELEAPLKICGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYVDRGKQSLE
                     TICLLLAYKIKYPENFFLLRGNHECASINRIYGFYDECKRRYNIKLWKTFTDCFNCLP
                     IAAIVDEKIFCCHGGLSPDLQSMEQIRRIMRPTDVPDQGLLCDLLWSDPDKDVQGWGE
                     NDRGVSFTFGAEVVAKFLHKHDLDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEF
                     DNAGAMMSVDETLMCSFQILKPADKNKGKYGQFSGLNPGGRPITPPRNSAKAKK"
BASE COUNT      288 a    400 c    393 g    286 t
ORIGIN
        1 gggcaaggag ctgctggctg gacggcggca tgtccgacag cgagaagctc aacctggact
       61 cgatcatcgg gcgcctgctg gaagtgcagg gttcgcgggc tggcaagaat gtacagctga
      121 cagagaacga gatccgcggt ctgtgcctga aatcccggga gatttttctg agccagccca
      181 ttcttctgga gctggaggca ccccttaaga tctgcggtga catacacggc cagtactacg
      241 acctttctgg actatttgag tatggcggtt tccctcccga gagcaactac ctctttctgg
      301 gggactatgt ggacagggga aagcagtcct tggagaccat ctgcctgctg ctggcctata
      361 agatcaagta ccccgagaac ttcttcctgc tccgtgggaa ccacgagtgt gccagcatca
      421 accgcatcta tggtttctac gatgagtgca agagacgcta caacatcaaa ctgtggaaaa
      481 ccttcactga ctgcttcaac tgcctgccca tcgcggccat agtggacgaa aagatcttct
      541 gctgccacgg aggcctgtcc ccggacctgc agtctatgga gcagattcgg cggatcatgc
      601 ggcccacaga tgtgcctgac cagggcctgc tgtgtgacct gctgtggtct gaccctgaca
      661 aggacgtgca gggctggggc gagaacgacc gtggcgtctc ttttaccttt ggagccgagg
      721 tggtggccaa gttcctccac aagcacgact tggacctcat ctgccgagca caccaggtgg
      781 tagaagacgg ctatgagttc tttgccaagc ggcagctggt gacacttttc tcagctccca
      841 actactgtgg cgagtttgac aatgctggcg ccatgatgag tgtggacgag accctcatgt
      901 gctctttcca gatcctcaag ccagccgaca agaacaaggg gaagtacggg cagttcagtg
      961 gcctgaaccc tggaggccga ccatcaccc caccccgcaa ttccgccaaa gccaagaaat
     1021 agcccccgca caccaccctg tgcccagat gatggattga ttgtacagaa atcatgctgc
     1081 catgctgggg gggggtcacc ccgaccccta aggcccacct gtcacgggga acatggagcc
     1141 ttggtgtatt tttcttttct ttttttaatg aatcaatagc agcgtccagt cccccagggc
     1201 tgcttcctgc ctgcaccctgc ggtactgtga gcaggatcct ggggccgagg ctgcagctca
     1261 gggcaacggc aggccaggtc gtgggtctcc agccgtgctt ggcctcaggc tggcagcccg
     1321 gatcctgggg caacccatct ggtctcttga ataaaggtca aagctgg
```

Table 6 (continued)

```
LOCUS       BOVPHO2A     1721 bp    mRNA            MAM       15-JUN-1988
DEFINITION  Bovine protein phosphatase type 2A catalytic subunit mRNA, complete
            cds.
ACCESSION   M16968
NID         g163515
KEYWORDS    phosphoprotein phosphatase 2A.
SOURCE      Bovine adrenal, cDNA to mRNA, clone pPBC-1.
  ORGANISM  Bos taurus
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Artiodactyla; Ruminantia; Pecora; Bovoidea;
            Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 1721)
  AUTHORS   Green,D.D., Yang,S.-I. and Mumby,M.C.
  TITLE     Molecular cloning and sequence analysis of the catalytic subunit of
            bovine type 2A protein phosphatase
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 84, 4880-4884 (1987)
  MEDLINE   87260893
COMMENT     Draft entry and printed copy of sequence for [1] kindly provided by
            M.C.Mumby, 09-SEP-1987.
FEATURES             Location/Qualifiers
     source          1..1721
                     /organism="Bos taurus"
                     /db_xref="taxon:9913"
     mRNA            <1..1721
                     /note="PP-C mRNA"
     CDS             104..1081
                     /note="protein phosphatase type 2A catalytic subunit"
                     /codon_start=1
                     /db_xref="PID:g163516"
                     /translation="MDEKVFTKELDQWIEQLNECKQLSESQVKSLCRRLKKSWTKESN
                     VQEVRCPVTVRGDVHGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLLVA
                     LKVRYRERITILRGNHESRQITQVYGFYDECLRKYGNANVWKYFTDLFDYLPLTALVD
                     GQIFCLHGGLSPSIDTLDHIRALDRLQEVPHEGPMCDLLWSDPDDRGGWGISPRGAGY
                     TFGQDISETFNHANGLTLVSRAHQLVMEGYNWCHDRNVVTIFSAPNYCYRCGNQAAIM
                     ELDDTLKYSFLQFDPAPAEASHMLLVVPQTTSCNEILNLYSIAMNHILT"
BASE COUNT      477 a    349 c    398 g    497 t
ORIGIN      13 bp upstream of SmaI site.
        1 tcacaaatac ccgggaacc gcggcggcgt gtgcgtgtgg ccgcgtgtgc ggcggcggcg
       61 cgggaggagc cgggagcggc agccggttcg ggcgggtggc atcatggacg agaaggtgtt
      121 caccaaggag ctggaccagt ggatcgagca gctgaacgag tgcaagcagc tgtctgagtc
      181 ccaggttaag agcctctgca gaaggctaaa gaaatcctgg acaaaagaat ccaatgtgca
      241 agaagttcga tgtccagtca ctgtccgtgg agatgtgcat gggcaatttc atgatctcat
      301 ggaactgttt agaattggtg gcaaatcacc agatacaaat tacttgttta tgggcgatta
      361 tgttgacaga ggatattatt cagtggaaac agttactctg cttgtagctc ttaaggttcg
      421 ttaccgtgaa cgtatcacca ttcttcgagg aaatcatgag agcagacaga tcacacaagt
      481 atatggtttc tacgatgagt gttttaaggaa atacggaaat gcaaatgttt ggaagtattt
      541 tacagacctt tttgactatc ttcctctcac tgccttggtg gatgggcaga tcttctgtct
      601 acatggtggc ctctcaccat ccatagatac actggatcac atcagagcac ttgatcgcct
      661 acaagaagtt cctcatgagg gtccaatgtg tgacttgctg tggtcagatc cagatgaccg
      721 tggaggttgg ggtatatctc ctcgaggagc tggttacacc tttgggcagg atattttctga
      781 gacatttaat catgccaatg gcctcacgtt ggtgtctaga gctcatcagc tggtgatgga
      841 gggatataac tggtgccatg accgaaatgt agtaacgatt ttcagtgctc caaactattg
      901 ttatcgttgt ggtaaccaag ctgcaatcat ggaacttgat gatactctaa aatactcttt
      961 cttgcagttt gacccagcac ccgcagaggc gagccacatg ttactcgtcg taccccagac
     1021 tacttcctgt aatgaaattt taaacttgta cagtattgcc atgaaccata tattgaccta
     1081 atggatatgg caagagcaac agtaactcca caagtgtcag agaatagtta acattcaaaa
     1141 aaacttgttt tcacacggac caaaaagatg tgccatataa aaatacaaag cctgtcatca
     1201 acagccgtga ccactttaga atgaaccagt tcattgcatg ctgaagcgac attgttggtc
```

Table 6 (continued)

```
1261 aagaaaccag tttctggcat agcgctattt gtagttactt tgctttctct gtgagactgc
1321 agataagatg taaacattaa cacctcgtga atacaattta acttccattt agctatagct
1381 ttactcagca tgactgtagg ataagaatag cagcaaacaa tcattggagc ttaatgaaca
1441 tttttaaaaa taagtaccaa ggcctccctt ctacttgtga gttttaaaat cgtttttgtt
1501 tattttcagg gtaccgttta atttaatttgt atgatttgtc tcgcatcagt ttatttttcc
1561 tctcaaatct agcctcatgt tgttcttttgt tactgtcaca acctggtgag ttgttttgaa
1621 tggaattgtt ttttttttc cctgctgtaa gatgatgtta ctgcacaaga gcactgcagt
1681 gtttttcata ataaacttgt gaactaagag atgaaaaagt c
```

(C2) Suppression of the protein phosphatase regulatory subunits

As a complementary approach to the one described in Section c.1, the expression of the regulatory subunits of either PP1 (inhibitor-1, inhibitor-2, or DARPP-32) or PP2A can be suppressed by one of the techniques described previously herein. For informational purposes, the DNA sequences of these substances is provided by Table 7 below.

Table 7

```
LOCUS       AI139158      481 bp    mRNA            EST       23-SEP-1998
DEFINITION  qc19d12.x1 Soares_fetal_heart_NbHH19W Homo sapiens cDNA clone
            IMAGE:1710071 3' similar to SW:IPP1_HUMAN Q13522 PROTEIN
            PHOSPHATASE INHIBITOR 1 ;, mRNA sequence.
ACCESSION   AI139158
NID         g3645130
KEYWORDS    EST.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
            Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 481)
  AUTHORS   NCI-CGAP http://www.ncbi.nlm.nih.gov/ncicgap.
  TITLE     National Cancer Institute, Cancer Genome Anatomy Project (CGAP),
            Tumor Gene Index
  JOURNAL   Unpublished (1997)
COMMENT
            Contact: Robert Strausberg, Ph.D.
            Tel: (301) 496-1550
            Email: Robert_Strausberg@nih.gov
            This clone is available royalty-free through LLNL ; contact the
            IMAGE Consortium (info@image.llnl.gov) for further information.
            Seq primer: -40m13 fwd. ET from Amersham
            High quality sequence stop: 459.
FEATURES             Location/Qualifiers
     source          1..481
                     /organism="Homo sapiens"
                     /note="Organ: heart; Vector: pT7T3D (Pharmacia) with a
                     modified polylinker; Site_1: Not I; Site_2: Eco RI; 1st
                     strand cDNA was primed with a Not I - oligo(dT) primer (5'
                     TGTTACCAATCTGAAGTGGGAGCGGCCGCATCTTTTTTTTTTTTTTTT 3'),
                     double-stranded cDNA was size selected, ligated to Eco RI
                     adapters (Pharmacia), digested with Not I and cloned into
                     the Not I and Eco RI sites of a modified pT7T3 vector
                     (Pharmacia). Library went through one round of
                     normalization to a Cot = 5. Library constructed by
                     M.Fatima Bonaldo. This library was constructed from the
                     same fetus as the fetal lung library, Soares fetal lung
                     NbHL19W."
                     /db_xref="taxon:9606"
                     /clone="IMAGE:1710071"
                     /clone_lib="Soares_fetal_heart_NbHH19W"
                     /sex="unknown"
                     /dev_stage="19 weeks"
                     /lab_host="DH10B (ampicillin resistant)"
BASE COUNT       89 a    123 c    118 g    151 t
ORIGIN
        1 ttgacctaac accaaattta tcacttttta aaaacaagag attttcccca aaagtgaagg
       61 aataagaaac aaatccggtg tccatgcatt cccaaactgc agtcttgatc ccaagatacc
      121 tcctcctctc tcagaccgag ttggctccct tggaatccag tggtggtata tgggttgagg
      181 gttcttttgt gctgggttcc ttactgcctc tctcgtgagt tttagggatg cattctgcag
      241 tttttttgc tgtcccagag gtgcccagcc ttgactccac ttctgtgtct gggatcccag
      301 gtgggcggga ctcctgggtt cctgtgctct cagcggcccc ctcaggttcc tctccttgct
      361 gctgttgccc caggtgatgt tcaaccatca tctggagctc tttcattgtg ggtgtgatcc
      421 ttgtcatctt cttccgttgc cgtggagaca ttgccaaagt ggacttgaga tgtgggttgg
      481 g
```

Table 7 (continued)

```
LOCUS       AA188560      439 bp    mRNA            EST       10-MAR-1998
DEFINITION  zp78f05.r1 Stratagene HeLa cell s3 937216 Homo sapiens cDNA clone
            626337 5' similar to SW:IPP2_HUMAN P41236 PROTEIN PHOSPHATASE
            INHIBITOR 2 ;, mRNA sequence.
ACCESSION   AA188560
NID         g1775788
KEYWORDS    EST.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
            Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 439)
  AUTHORS   Hillier,L., Allen,M., Bowles,L., Dubuque,T., Geisel,G., Jost,S.,
            Krizman,D., Kucaba,T., Lacy,M., Le,N., Lennon,G., Marra,M.,
            Martin,J., Moore,B., Schellenberg,K., Steptoe,M., Tan,F.,
            Theising,B., White,Y., Wylie,T., Waterston,R. and Wilson,R.
  TITLE     WashU-NCI human EST Project
  JOURNAL   Unpublished (1997)
COMMENT
            Contact: Wilson RK
            Washington University School of Medicine
            4444 Forest Park Parkway, Box 8501, St. Louis, MO 63108
            Tel: 314 286 1800
            Fax: 314 286 1810
            Email: est@watson.wustl.edu
            This clone is available royalty-free through LLNL ; contact the
            IMAGE Consortium (info@image.llnl.gov) for further information.
            Insert Length: 1296   Std Error: 0.00
            Seq primer: -28M13 rev2 from Amersham
            High quality sequence stop: 368.
FEATURES              Location/Qualifiers
     source           1..439
                      /organism="Homo sapiens"
                      /note="Vector: pBluescript SK-; Site_1: EcoRI; Site_2:
                      XhoI; Cloned unidirectionally.  Primer: Oligo dT. HeLa S3
                      epithelioid carcinoma cells grown to semi-confluency
                      without induction. Average insert size: 1.5 kb; Uni-ZAP XR
                      Vector. ~5' adaptor sequence: 5' GAATTCGGCACGAG 3' ~3'
                      adaptor sequence: 5' CTCGAGTTTTTTTTTTTTTTTT 3'"
                      /db_xref="GDB:5045035"
                      /db_xref="taxon:9606"
                      /clone="626337"
                      /clone_lib="Stratagene HeLa cell s3 937216"
                      /sex="female"
                      /dev_stage="HeLa S3 cell line"
                      /lab_host="SOLR (kanamycin resistant)"
BASE COUNT      154 a     89 c    112 g     83 t       1 others
ORIGIN
        1 gacttcctct atggtggcgt cggcgaacag ccccgcggga atgtcgacga ggagctgagc
       61 aaaaaatccc agaagtggga tgaaatgaac atcttggcga cgtatcatcc agcagacaaa
      121 gactatggtt taatgaaaat agatgaacca agcactcctt accatagtat gatgggggat
      181 gatgaagatg cctgtagtga caccgaggcc acttgaagcc atggcgccag acatctttag
      241 ccaggaaatt agctgcagct gaaggcttgg agccaaagta tcggattcag gaacaagaaa
      301 gcagtggaga ggaggatagt gacctctcac ctgaagaacg agaaaaaaag cgacaatttg
      361 aaatgaaaag gaagcttcac tacaatgaag gactcaatat caaactagcc agacanttaa
      421 tttcaaaaga cctacatga
```

Table 7 (continued)

```
LOCUS       HSU60823      750 bp    mRNA            PRI       11-JUL-1996
DEFINITION  Human potent heat-stable protein phosphatase 2A inhibitor I1PP2A
            mRNA, complete cds.
ACCESSION   U60823
NID         g1408223
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 750)
  AUTHORS   Li,M., Makkinje,A. and Damuni,Z.
  TITLE     Molecular identification of I1PP2A, a novel potent heat-stable
            inhibitor protein of protein phosphatase 2A
  JOURNAL   Biochemistry 35 (22), 6998-7002 (1996)
  MEDLINE   96240314
REFERENCE   2  (bases 1 to 750)
  AUTHORS   Li,M., Makkinje,A. and Damuni,Z.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-JUN-1996) Cellular & Molecular Physiology,
            Pennsylvania State University College of Medicine, 500 Univeristy
            Drive, Hershey, PA 17033, USA
FEATURES             Location/Qualifiers
     source          1..750
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /tissue_type="kidney"
     CDS             1..750
                     /codon_start=1
                     /product="potent heat-stable protein phosphatase 2A
                     inhibitor I1PP2A"
                     /db_xref="PID:g1408224"
                     /translation="MEMGRRIHLELRNRTPSDVKELVLDNSRSNEGKLEGLTDEFEEL
                     EFLSTINVGLTSIANLPKLNKLKKLELSDNRVSGGLEVLAEKCPNLTHLNLSGNKIKD
                     LSTIEPLKKLENLKSLDLFNCEVTNLNDYRENVFKLLPQLTYLDGYDRDDKEAPDSDA
                     EGYVEGLDDEEEDEDEEEYDEDAQVVEDEEDEDEEEEGEEEDVSGEEEEDEEGYNDGE
                     VDDEEDEEELGEEERGQKRKREPEDEGEDDD"
BASE COUNT      260 a    130 c    229 g    131 t
ORIGIN
        1 atggagatgg gcagacggat tcatttagag ctgcggaaca ggacgccctc tgatgtgaaa
       61 gaacttgtcc tggacaacag tcggtcgaat gaaggcaaac tcgaaggcct cacagatgaa
      121 tttgaagaac tggaattctt aagtacaatc aacgtaggcc tcacctcaat cgcaaacttа
      181 ccaaagttaa acaaacttaa gaagcttgaa ctaagcgata acagagtctc agggggcctg
      241 gaagtattgg cagaaaagtg tccgaacctc acgcatctaa atttaagtgg caacaaaatt
      301 aaagacctca gcacaataga gccactgaaa aagttagaaa acctcaagag cttagacctt
      361 ttcaattgcg aggtaaccaa cctgaacgac taccgagaaa atgtgttcaa gctcctcccg
      421 caacttacat atctcgacgg ctatgaccgg gacgacaagg aggcccctga ctcggatgct
      481 gagggctacg tggagggcct ggatgatgag gaggaggatg aggatgagga ggagtatgat
      541 gaagatgctc aggtactgga agacgaggag gacgaggatg aggaggagga aggtgaagag
      601 gaggacgtga gtggagagga ggaggaggat gaagaaggtt ataacgatgg agaggtagat
      661 gacgaggaag atgaagaaga gcttggtgaa gaagaaaggg gtcagaagcg aaaacgagaa
      721 cctgaagatg agggagaaga tgatgactaa
```

Table 7 (continued)

```
LOCUS       BOVDARPP32   1691 bp    mRNA            MAM       15-DEC-1994
DEFINITION  Bos taurus (clone pTKD7) dopamine and cyclic AMP-regulated neuronal
            phosphoprotein (DARPP-32) mRNA, complete cds.
ACCESSION   M27444
NID         g602437
KEYWORDS    phosphoprotein.
SOURCE      Bos taurus calf brain (caudate nucleus) cDNA to mRNA.
  ORGANISM  Bos taurus
            Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
            Vertebrata; Eutheria; Artiodactyla; Ruminantia; Pecora; Bovoidea;
            Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 1691)
  AUTHORS   Kurihara,T., Lewis,R.M., Eisler,J. and Greengard,P.
  TITLE     Cloning of cDNA for DARPP-32, a dopamine- and cyclic AMP-regulated
            neuronal phosphoprotein
  JOURNAL   J. Neurosci. 8 (2), 508-517 (1988)
  MEDLINE   88117716
COMMENT     On Dec 16, 1994 this sequence version replaced gi:341693.
FEATURES             Location/Qualifiers
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                     /dev_stage="calf"
                     /clone="pTKD7"
                     /tissue_type="brain (caudate nucleus)"
     gene            342..1680
                     /gene="DARPP-32"
     CDS             342..950
                     /gene="DARPP-32"
                     /note="dopamine and cyclic AMP regulated neuronal protein"
                     /codon_start=1
                     /product="phosphoprotein"
                     /db_xref="PID:g602438"
                     /translation="MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLSEH
                     SSPEEEASPHQRASGEGHHLKSKRSNPCAYTPPSLKAVQRIAESHLQSISNLGENQAS
                     EEEDELGELRELGYPREEEEEEEEEDEEEEEDSQAEVLKGSRGSAGQKTTYGQGLEGP
                     WERPPPLDGPQRDGSSEDQVEDPALNEPGEEPQRPAHPEPGT"
     polyA_signal    1675..1680
                     /gene="DARPP-32"
     polyA_site      1691
                     /gene="DARPP-32"
BASE COUNT      373 a    530 c    493 g    295 t
ORIGIN
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       61 gagagacaca gacagagcga gggaaggagg gccagagacc agccccgcga gcccgaggtg
      121 cggccccagg ggaccgggca cccaggagcc ccagagccgc gagccggccg ccccccaccc
      181 ccgcgcgccc tccccccgcc ggcgggtatt tttatctgtg cgtgaacagc cctccagctc
      241 ctctctgcca cactcaaccc gctgccgccg cggccccgag cagcgcgggg gagcggagcg
      301 ggatcgcagc ccgagacccc acgacgcgcg ccccgcccgc catggacccc aaagaccgca
      361 agaagatcca gttctccgtg cccgcgcccc ccagccagct cgacccccgc caggtggaga
      421 tgatccggcg caggagacca acccctgcca tgctgttccg gctctcagag cactcctcac
      481 cagaggagga ggcctcaccc caccagagag cctcaggaga ggggcaccac ctcaagtcga
      541 agagatccaa tccttgtgcc tacacacccc cctcgctgaa agccgtccag cgcattgctg
      601 agtctcacct gcagtccatc agcaacctgg gtgagaacca ggcctcggag gaggaggatg
      661 agctgggggg gctgcgggaa ctgggctacc caagagagga agaggaggag gaagaggagg
      721 aggatgaaga ggaggaggag gacagccagg cggaagtcct gaagggcagc agggggtctg
      781 ctgggcagaa gacaacttat ggccaaggtc tggagggtcc ctgggagcgc ccgcctcctc
      841 tggatgggcc ccagagagac ggaagctctg aggaccaagt ggaagaccca gcattgaatg
```

Table 7 (continued)

```
 901 aaccccgggga ggagccacag cgccctgccc accctgagcc tggcacatag gcacccagcc
 961 ctgcatcctcc tggaaggaag tgggaggaag cattgatgtt ctccagaaac ccattctgtt
1021 cacaccctat tttgtaccct gcttctcact tgctagggct gcggcttctg acttttagaa
1081 gactaaggct ggtctgtgtt tgcttgtctg cccaacttcg ctgatcccag agtccctggg
1141 cacttgctgc ctgatgccta ccccctgccag tcattccccc atacacccag caggaggtgg
1201 gatgggagag cttgcattgg gaaatccagt aaatgggggga caaagattca tccttcacaa
1261 ttctactccc tagaccctct cccctgggcg taggaaacca cagggcagga ccctaagatc
1321 tggggaaaag ggatactgag aacttgtaag tgcccataga tcttttctcca tccccctgggc
1381 aattccaagt catcaccccc tcactgcctt ctaccagggc ccagaattca ggcatctttt
1441 ccacggcctc agcttttggt aaatcttccc cttatcacct gctccccagc ctgggtgcct
1501 ggaagatcga ctggcagaga ctgctttgct gcattttatg tgtgctttga tgccaggaat
1561 gccacctagt ataataagtc cttaggggcg cacatggtgg gggagccaag cctccttgt
1621 cctccagctg cctgtccccc ttcccctctt ccctgactcc cggcctgaac ctgtaataaa
1681 tctttgtaaa t
```

## IV. Routes of Administration, Formulations, and Dosages

In general, the compositions and agents described herein for use in the methodology can be administered in any appropriate carrier for oral, topical, or parenteral administration under in-vivo conditions to a living host subject. The compositions can be introduced by any means that brings access to the vascular endothelial cells within the tissues and organs in the host body. The dosage administered will, of course, vary and be dependent upon the age, health, and weight of the intended recipient; the kind of concurrent treatment, if any; the frequency of treatment, and the degree of the therapeutic effect desired.

If the compositions and agents are to be administered topically, they can be admixed in a concentration range in a pharmaceutically inert topical carrier such as a gel, an ointment, a lotion, or a cream and include such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils. Other topical carriers are represented by liquid petrolatum, isopropyl palmitate and the like. In addition, minerals such as anti-oxidants, viscosity stabilizers and the like may be added if and when necessary.

If the compositions and agents are to be given parenterally, these compositions will be prepared in sterile form; in multiple or single dose formats; and dispersed in a fluid carrier such as sterile physiological saline, or 5% dextrose solutions commonly used with injectables. In addition, other modes of administration such as perfusion, intravenous injection, and lavage may be advantageously employed as well.

## V. Experiments And Empirical Data

To demonstrate the merits and value of the present invention, a series of planned experiments and empirical data are presented below. It will be expressly understood, however, that the experiments described and the results provided are merely the best evidence of the subject matter as a whole which is the invention; and that the empirical data, while limited in content, is only illustrative of the scope of the invention envisioned and claimed.

## Experimental Series I

This series of experiments was performed to determine whether the syndecan-4 molecule is itself subject to phosphorylation; and also to determine whether such phosphorylation is affected by the binding of a growth factor (bFGF) to its receptors on the cell surface.

**Experimental Procedures:**

Materials

Calyculin, chelerythrine, PMA, and bFGF were purchased from Sigma. Gö 6976 was purchased from Calbiochem (La Jolla, CA). Chelerythrine, PMA, and Gö 6976 were dissolved in $Me_2SO$.

Isolation of Syndecan-4 Core Proteins

NIH 3T3 cells (American Type Culture Collection, Bethesda, MD) were grown to confluence in 100-mm plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Life Technologies Inc.) at 37 °C in a 5% $CO_2$ humidified atmosphere. The cells were harvested by scraping in 1 ml of lysis buffer (150 mm NaCl, 20 mM NaF, 20 mM $Na_4P_2O_7$, 5 mM EDTA, 5 mM EGTA, 1mM $Na_3VO_4$, 1 mM phenylethylsulfonyl fluoride, 1% Triton X-100, 50 mM HEPES, pH 7.4). The lysate was cleared by centrifugation at 9000 x *g* for 30 min. and then subjected to DEAE-chromatography as described by Scworak et al., J. Biol. Chem. 269: 21204-21214 (1994)]. The eluates were dialyzed twice against 10 mM $NH_4HCO_3$, 1 mM β-mercaptoethanol and concentrated by evaporation under vacuum. The concentrated samples were resuspended in 50 μl of digestion buffer (50 mM NaCl, 4 mM $CaCl_2$, 20 mM Tris, pH 7.4) and GAG chains were cleaved off the proteoglycan core proteins by 4 h of incubation in a mixture of 0.06 unit of chondroitinase ABC and 1 unit each of heparinases I, II, and III (Sigma) at 37 °C.

Radiolabeling of Cultured Cells

Confluent NIH 3T3 cells were washed twice in phosphate-free DMEM and incubated for 24 h at 37 °C in a 5% $CO_2$ humidified atmosphere in phosphate-free DMEM supplemented with 0.5% fetal bovine serum. The cells were washed twice with methionine, phosphate, and serum-free DMEM and incubated for 6 h in the same medium, supplemented with 400 μCi/ml [$^{35}$S]methionine (New England Nuclear, Boston, MA). At the onset of the last 2 h of incubation, 500 μCi/ml [$^{32}$P]orthophosphoric acid (New England Nuclear) was added to the medium.

Immunoprecipitation of Cytoplasmic and Ectoplasmic Syndecan-4 Domains

Cells were washed with PBS (137 mL NaCl, 10 mM $Na_2HPO_4$, 3.6 mM KCl, 1.8 mM $KH_2PO_4$, pH 7.4), dissociated by 0.05% trypsin, 0.5 mM EDTA (Life Technologies, Inc.) in PBS for 10 min at 22 °C, and sedimented by 2000 x *g* centrifugation at 4 °C for 5 min. The syndecan-4 ectoplasmic domain was immunoprecipitated from 0.5 ml of medium collected before cell trypsinization or from 0.5 ml of supernatant of the latter centrifugation. The cytoplasmic tail was immunoprecipitated from the pellet after a 30-min extraction at 4 °C in 0.5 mil of lysis buffer supplemented with 100 μM leupeptin, 2 μM pepstatin, and 10 nM okadaic acid (Sigma). Total protein concentrations in each fraction were measured by spectrophotometry at 595 nm (DU 640, Beckman, Fullerton, CA) of an aliquot developed for 10 min in Protein Assay Dye Reagent (Bio-Rad). Bovine serum albumin (Life Technologies Inc.) was used as standard.

The medium, trypsinization supernatant, and extracted pellet fractions were precleared by adding 30 μl of 1:1 (v/v) slurry of protein G plus/protein A-agarose beads (Calbiochem), and 10 μl of nonimmune rabbit serum (Life Technologies Inc.). After a 2-h incubation at 4 °C in rotating tubes, the beads were sedimented by 5 min, 5000 x *g* centrifugation at 4 °C. The cleared samples were supplemented with 40 μl of 1:1 (v/v) slurry of the above beads and 10 μl of rabbit polyclonal antiserum (syndecan-4 ectoplasmic domain-specific antiserum was added to the medium and trypsinization supernatant samples, and cytoplasmic tail-specific antiserum was added to the extracted pellet fraction) and incubated in rotating tubes for 18 h at 4 °C. The agarose beads were collected by centrifugation as above,

washed three times in heparinase digestion buffer, and resuspended in 40 μl of digestion buffer, and the GAG chains of the bead-attached ectoplasmic domains from the medium and from the trypsinization-supernatant were cleaved as above. The ectoplasmic and cytoplasmic tails were dissociated from the beads by a 10-min incubation in SDS buffer at 95 °C, and the beads were sedimented by a 5 min, 13,000 x *g* centrifugation at 4 °C.

Electrophoresis, Transfer, Autoradiography and Immunoblotting

Immunoprecipitated, full-length syndecan-4 core proteins were resuspended in Laemmli sample buffer (2% SDS, 10% glycerol), 0.5% β-mercaptoethanol, 0.004% bromphenol blue, 50 mM Tris-HCl, pH 6.8) resolved by SDS-PAGE on a 10% slab gel, and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA) for 12 h at 25 mA in 150 mM glycine, 20 mM Tris-HCl, and 20% methanol. The ectoplasmic and cytoplasmic syndecan-4 domains were resolved on a 15% slab gel and transferred for 90 min at 20 mA in 150 mM glycine, 20 mM Tris-HCl, and 30% methanol to a low porosity PVDF membrane (Immobilon-$P^{SQ}$, Millipore). Radiolabeled bands detected by exposure to film (XAR, Kodak, Rochester, NY) were excised, and their radioactivity was measured in both the $^{32}P$ and $^{35}S$ spectra by scintillation counting (LS 6000IC, Beckman, Fullerton, CA). In some cases, the same membranes were used for immunoblotting prior to band excision.

After blocking in PBS containing 5% nonfat milk powder for 1 h at 22 °C, the membrane was incubated in the same solution supplemented with 1:3000 (v/v) dilution of either ectoplasmic or cytoplasmic tail-specific antiserum for 2 h, washed with PBS, and incubated for 1 h in 5% milk powder-PBS containing 1:2000 diluted goat anti-rabbit IgG conjugated to peroxidase (Vector Laboratories, Burlingame, CA). The secondary antibody was detected, after no additional PBS wash, by chemiluminescence (Western Blot, Chemiluminescence Reagent Plus, New England Nuclear). Molecular weights were estimated by comparison with the electrophoretic mobility of standards (Kaleidoscope Prestained Standards, Bio-Rad).

Densitometry of digitized images of immunoprobed membranes (ScanJet 4c, Hewlett Packard) was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Thin-Layer Chromatography

Bands excised from PVDF membranes were hydrolyzed for 75 min in 6N HCl at 110 °C. Solvent was evaporated under vacuum, and the sediment was washed thrice with $H_2O$. The sediment was resuspended in 5 $\mu$l of $H_2O$ after the third evaporation, applied to a thin layer cellulose acetate plate (Sigma-Aldrich), and underwent electrophoresis at 1000 V in 5% acetic acid, 0.5% pyridine, pH 3.0. The radiolabeled phosphoamino acids were detected by phospho-luminescence (PhosphorImager, Molecular Dynamics). Phosphorylated, unlabeled Ser, Thr, and Tyr (Sigma) were used as standards and were detected by spraying with ninhydrin.

**Experiment 1**

To determine the presence and extent of phosphorylation of the syndecan-4 cytoplasmic tail, full-length heparan and chondroitin sulfate-carrying core proteins were isolated from serum-starved, $^{32}$P-labeled NIH 3T3 cells. The results are shown by Figs. 1A and 1B respectively.

Fig. 1 generally shows the detection of syndecan-4 core protein basal phosphorylation and identification of serine phosphorylation. Lane 1 of Fig. 1A shows an autoradiograph of fibroblast proteoglycans resolved by 10% SDS-PAGE and transferred to a PVDF membrane; Lane 2 of Fig. 1A shows a Western immunoblast of the same membrane shown in lane 1. In comparison, Fig. 1B shows a phospholuminescence image of the acid-hydrolyzed, TLC-separated syndecan-4 band (*syn-4*) shown in Fig. 1. Phosphoamino acids were identified by comparison with the electrophoretic mobility of nonradiolabeled standards: *P-Ser*, Ser(P); *P-Thr*, Thr(P); *P-Tyr*, Tyr(P).

The results of the autoradiography of NIH 3T3 GAG-lysed core proteins is shown in Fig. 1A (lane 1). To identify the syndecan-4 band, the autoradiographed

membrane was probed with an antiserum specific to the cytoplasmic tail of the syndecan-4 core protein. The immunoblotting highlighted a single band that ran at an approximate molecular mass of 36 kDa (Fig. 1A, lane 2). A similar syndecan-4 electrophoretic mobility lower than its predicted molecular mass of 20 kDa was observed before with the same antiserum. As illustrated in Fig. 1A, the antiserum-detected band superimposed precisely on the second band from the bottom in the autoradiograph.

Phosphoamino acid analysis of the syndecan-4 band produced a single, intensely labeled spot that corresponded to the electrophoretic mobility of phosphorylated serine as shown by Fig. 1B. The syndecan-4 core protein sequence contains multiple serines [16 in the human syndecan-4 and 15 in the rat] - all but one of which are located in the ectoplasmic domain.

**Experiment 2:**

To determine which domain contains the phosphorylated serine, the susceptibility of the ectoplasmic domain of the syndecans to trypsinization was exploited. Thus the core protein of syndecan-4 was cleaved at the cell surface concurrently with the trypsin dissociation of the $^{32}P$-labeled, adherent cells from the culture plates. The results are visually seen in Fig. 2.

Fig. 2 shows the localization of syndecan-4 core protein phosphorylation to the cytoplasmic tail. Lane 1 of Fig. 2A shows an autoradiograph of Triton X-100 soluble cell fraction immunoprecipitated with syndecan-4 (*syn-4*) cytoplasmic tail-specific antiserum. Lane 2 of Fig. 2A shows a Western immunoblot of the same membrane shown in lane 1, using syndecan-4 cytoplasmic tail-specific antiserum. Lane 1 Fig. 2B shows Triton X-100 soluble cell fraction immunoprecipitated with syndecan-4 cytoplasmic tail-specific antiserum. Lane 2 of Fig. 2B shows the trypsinized ectoplasmic proteins fraction immunoprecipitated with antiserum specific to the syndecan-4 ectoplasmic domain. Lane 3 of Fig. 2B shows the cell culture medium immunoprecipitated and processed as the sample in lane 2. Autoradiography exposure times were identical for the three samples.

Accordingly, by analogy with syndecan-1, the trypsinization site is most likely between $Arg^{147}$ and $Thr^{148}$ preceding the transmembrane domain. Following trypsinization and detergent extraction, the cleaved syndecan-4 fragment was isolated by immunoprecipitation with the cytoplasmic tail-specific antiserum, which recognizes a 14-residue cytoplasmic sequence. As with the full-length core protein, the $^{32}P$-labeled immunoprecipitate was separated by SDS-PAGE and transferred to a membrane. The band routinely detected in the autoradiographs of these membranes migrated at an approximate molecular mass of 5 kDa, slightly less than the predicted 7-kDa size of the fragment encompassing the trypsinized transmembrane and cytoplasmic tails (Fig. 2A). This lower apparent molecular mass may have resulted from partial degradation during the isolation process or may reflect a higher electrophoretic mobility than the molecular mass standard used for estimating the band size.

To verify the identity of this band, the same membrane was reprobed with the antiserum that recognizes the cytoplasmic tail. The immunoblotted band overlapped the $^{32}P$-labeled one (Fig. 2A), confirming that the latter is comprised of the $Ser^{183}$-phosphorylated cytoplasmic tail of syndecan-4.

To rule out phosphorylation of additional serines in the syndecan-4 core protein outside the cytoplasmic tail, the phosphorylation was examined in three different fractions: (a) the medium, which could contain shed ectoplasmic syndecan-4; (b) the supernatant of the sedimented trypsinized cells, containing the cleaved ectoplasmic domain; and (c) the detergent-soluble fraction extracted from the pellet of the sedimentation, containing the transmembrane and cytoplasmic domains. The ectoplasmic domain of syndecan-4 was immunoprecipitated from the first two fractions with an antiserum specific to this domain; and the third fraction was immunoprecipitated with the cytoplasmic tail-specific antiserum. Auto-radiography of the SDS-PAGE-separated fractions revealed a single band in the cytoplasmic fraction lane. No radioactive bands were detected in the lanes of the other two fractions (Fig. 2B). This clearly localizes the phosphorylation to the single serine residue in the cytoplasmic tail of the core protein of syndecan-4.

**Experiment 3:**

To determine the stoichiometry of the basal phosphorylation of syndecan-4, as well as the effect of bFGF and of other compounds on this phosphorylation, the cells were doubly labeled with [$^{35}$S]methionine and [$^{32}$P]orthophosphoric acid. The syndecan-4 carboxyl-terminal proteolytic fragment produced by trypsinization between $Arg^{147}$ and $Thr^{148}$ (referring to the rat sequence numbering) contains a single methionine ($Met^{176}$). Because we have shown above that the phosphorylation of syndecan-4 occurs at a single $Ser^{183}$, the molar ratio of $^{32}P/^{35}S$, as calculated from their specific activities, should be equivalent to the ratio of mol $P_i$/mol syndecan-4, assuming the two radio probes have similar incorporation efficiencies. Because this quantitation method is radiometric, the result is independent of the absolute amounts of protein processed. Using this approach, the stoichiometry of the basal phosphorylation of syndecan-4 in cells starved for 24 h in 0.5% serum, followed by 6 h of serum-free starvation, was 0.31 ± 0.12 (mean ± S.D., n = 5) of mol $P_i$/mol syndecan-4.

**Experiment 4:**

The participation of the syndecan ectoplasmic domain in bFGF binding raises the question whether this binding is accompanied by intracellular modifications of syndecan-4, such as phosphorylation of its cytoplasmic tail. The results of Fig. 3 reveal the true answer.

Fig. 3 shows the effects of bFGF and calyculin on syndecan-4 cytoplasmic tail phosphorylation. Fig. 3A shows the cumulative results of the effects of bFGF (n = 4), calyculin (*cal*, *n* = 3), and bFGF together with calyculin (*bFGF+cal*, *n* = 3) on syndecan-4 cytoplasmic tail phosphorylation, relative to untreated control cells (cont). Bars denote standard deviation. The inset shows autoradiographs of cell lysates immunoprecipitated with syndecan-4 cytoplasmic tail-specific antiserum. Lane 1 represents bFGF-untreated cells. Lane 2 represents cells treated with 10 ng/ml bFGF. Lane 3 represents cells treated concurrently with 10 ng/ml bFGF and 5 nM calyculin. Arrow denotes the syndecan-4 band.

Fig. 3B reveals the dependence of syndecan-4 cytoplasmic tail phosphorylation on bFGF concentration. Phosphorylation stoichiometry was calculated as the ratio of $^{32}P/^{35}S$ counts of the syndecan-4 bands excised from PVDF membranes. Inset shows immunoblotted syndecan-4 bands from control and bFGF-treated (10 ng/ml) cells. Cell lysates containing equal amounts of total protein were applied in each lane.

Experimentally, treatment with 10 ng/ml of bFGF during the last 5 h of the serum-free starvation decreased the phosphorylation stoichiometry of syndecan-4 to $0.16 \pm 0.02$ ($n = 5$), approximately half its basal level (Fig. 3A). Larger bFGF dosages of 20 and 30 ng/ml further decreased the phosphorylation stoichiometry of syndecan-4 to $0.12 \pm 0.06$ ($n = 3$), but this decrease was not statistically different from the effect of 10 ng/ml bFGF (Fig. 3B).

To test for the possible involvement of a phosphatase in the bFGF-induced decrease of syndecan-4 phosphorylation, phosphatase 1/2A inhibitor calyculin (5 nM) was applied to bFGF (10 ng/ml)-treated cells. Calyculin countered the effect of bFGF, maintaining the syndecan-4 phosphorylation at its basal level (Fig. 3B). Moreover, when the same calyculin dose was applied to cells in the absence of bFGF, syndecan-4 phosphorylation was increased more than 2.5-fold relative to the basal level.

If, contrary to our assumption, the incorporation efficiency of $^{35}S$ is higher than that of $^{32}P$, the bFGF-induced decrease in syndecan-4 phosphorylation could solely result from bFGF up-regulation of syndecan-4 synthesis. To address this possibility, the syndecan-4 expression levels in control and in bFGF-treated cells (processed identically to those in the phosphorylation assays) were compared by immunoblotting cell lysates containing equal amounts of total protein. The syndecan-4 bands, which similar to immunoprecipitated samples (Fig. 2A) ran at an approximate molecular mass of 5 kDa, were detected with the antiserum specific to the ectoplasmic domain; and the amount of protein in each band was quantified by densiometry. In cells treated by 10 and by 30 ng/ml bFGF, the level of syndecan-4 expression was 85% (Fig. 3B, inset) and 93% of the control cells, respectively.

**Experiment 5:**

The possible involvement of PKC in syndecan-4 phosphorylation was then investigated. The experiment data is provided by Fig. 4.

Fig. 4 reveals the effects of PKC activation and inhibition on syndecan-4 cytoplasmic tail phosphorylation. Fig. 4A shows a phospholuminescence image of acid-hydrolyzed, TLC-separated, syndecan-4 (*syn-4*) bands excised from PVDF membranes of control cells treated by $Me_2$-SO alone (Lane 1) and from cells treated with 0.5 $\mu$M PMA (lane 2). Fig. 4B shows autoradiographs of immunoprecipitates from control (lane 1) and PMA-treated cells (lane 2) resolved by SDS-PAGE and transferred to a PVDF membrane. Fig. 4C shows the dependence of syndecan-4 phosphorylation stoichiometry on chelerythrine concentration. Chelerythrine was applied to the cells together with 0.5 $\mu$M PMA. These results are representative of two experiments.

Procedurally, to up-regulate PKC, cells were treated with the PKC-activating phorbol ester PMA (0.5 $\mu$M) during the last 5 h of the serum-free starvation. This treatment increased only the $Ser^{183}$ phosphorylation of syndecan-4, without having a detectable effect on the phosphorylation of threonine or trypsine residues in the cytoplasmic tail (Fig. 4A). The stoichiometry of the phosphorylation of syndecan-4 in the PMA-treated cells was $0.81 \pm 0.33$ ($n = 3$), close to 3-fold higher than the basal level. This result indicates that syndecan-4 is either a direct or an indirect PKC substrate.

To further examine the role of PKC in syndecan-4 phosphorylation, the PKC-specific inhibitor chelerythrine was applied to PMA-stimulated cells. The phosphorylation of syndecan-4 started to decline at chelerythrine concentrations above 1.5 $\mu$M and was reduced to an undetectable level at 6 $\mu$M chelerythrine (Fig. 4, B and C). The latter concentration is less than 10% of the $IC_{50}$ of chelerythrine for the inhibition of protein tryosine kinases. Although supporting the role of PKC in the phosphorylation of syndecan-4, these results do not identify the specific isozyme involved, because both PMA and chelerythrine affect all the four known calcium-dependent cPKCs, as well as the five calcium-independent nPKCs.

To further narrow down the group of possible PKC isozymes, the indolocarbazole Gö 6976, which inhibits calcium-dependent PKC isozymes, was applied to PMA (0.5 $\mu$M)-treated cells. The phosphorylation of syndecan-4 was not reduced, however, by Gö 6976 concentrations up to 100 nM, more than 10-fold its $IC_{50}$ for cPKC (data not shown). It is likely, therefore, that the syndecan-4 cytoplasmic tail is phosphorylated by one of the nPKC isozymes.

**Conclusions:**

1. The cytoplasmic tail of syndecan-4 is phosphorylated in cultured fibroblasts and that the extent of its phosphorylation is determined by activities of a nPKC enzyme and a bFGF-activated phosphatase. The phosphorylation site was localized to $Ser^{153}$, immediately upstream of a nine-amino acid segment involved in binding to and activation of PKC$\alpha$.

2. The experimental series showed a relatively high degree of syndecan-4 phosphorylation in growth-arrested cells, which could be further increased by treatment with PMA or decreased by bFGF. Because $Ser^{183}$ is part of an invariant seven-residue sequence (KKDEGSY), these findings may be relevant to all four members of the syndecan family.

3. The PMA-induced increase in the phosphorylation of syndecan-4 and its decrease by chelerythrine strongly suggest the involvement of PKC in this phosphorylation. In agreement with this observation, we were unable to suppress the PMA-induced phosphorylation of syndecan-4 by a cPKC-specific inhibitor, pointing to the participation of a nPKC isozyme in the phosphorylation.

4. A phosphatase inhibitor reversed the bFGF-induced reduction in syndecan-4 phosphorylation observed in our study. This suggests that bFGF binding up-regulates a phosphatase and/or down-regulates a kinase involved in controlling the level of $Ser^{183}$ phosphorylation.

## Experimental Series II

These experiments are directed to investigating the effect of phosphorylation of the cytoplasmic tail of syndecan-4 upon its interaction with phosphatidylinositol 4,5-biphosphate (hereinafter "$PIP_2$) which binds directly to the cytoplasmic tail of syndecan-4 and facilitates its multimerization; as well as its capacity to activate Protein Kinase C α. The involvement of $PIP_2$ for binding and multimerization has been reported previously in the scientific literature [Oh et al., J. Biol. Chem. 272: 11805-11811 (1997); Oh et al., J. Biol. Chem. 273: 10624-10629 (1998); and Lee et al., J. Biol. Chem. 273: 13022-13029 (1998)]. However, the functional effects of phosphorylating the cytoplasmic region of the syndecan-4 and its effects on the molecular properties and signaling activity have not yet been elucidated.

**Experimental Procedures:**

Materials

Phosphatidylinositol 4,5-biphosphate ($PIP_2$), phosphatidylserine (PS), and diolein were purchased from Sigma. Recombinant PKCα and PKCδ were synthesized and prepared as described in Nishikawa et al., J. Biol. Chem. 272: 952-960 (1997). PKCβ1 optimal substrate peptide (FKLKRKGSFKKFA) was purchased from Tufts University Medical School (Boston, MA). A 28 amino acid-long syndecan-4 cytoplasmic tail peptide (S4c) (RMKKKDEGSYDLG KKPIYKKAPTNEFYA) was synthesized by Genemed Synthesis (South San Francisco, CA). A similar peptide with a phosphorylated Ser (S4c-P) was synthesized by the Biopolymers Laboratory, Harvard Medical School (Boston, MA).

$PIP_2$ Binding Assay

$PIP_2$ (from Sigma, dissolved at 2 mg/ml in 20 parts $CHCl_3$, 9 parts MeOH, 1 part $H_2O$, 0.1 part 1N HCl) was dried under $N_2$ and sonicated for 5 min in ice-cold $H_2O$ at a final concentration of 1 mg/ml. Syndecan-4 cytoplasmic tail peptides S4c or S4c-P (100 μM) were incubated on ice for 30 min with the indicated concentrations of $PIP_2$ in 10 mM Tris-HCl (pH 7.5), 75 mM KCl,

0.5 mM DTT, in aliquots of 100 μl. The samples were layered on 30 kDA-molecular-mass cutoff cellulose filters (Ultrafree-MC, Millipore, Bedford, MA), and spun at 2000 *g* for 1 min, following the method described in Haarer et al., Mol. Cell Biol. 13: 7864-7873 (1993). The samples (40 μl of each in Laemmli sample buffer, 2% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 0.004% bromophenol blue, 50 mM Tris-HCl, pH 6.8) were resolved by SDS-PAGE on 16.5% Tris-tricine gels (BioRad Laboratories, Hercules, CA). Gels were stained with Coomassie Brilliant Blue G-250 (BioRad Laboratories), and images of the stained bands were digitized (DeskScan II on ScanJet 4c, Hewlett Packard) and quantitated by densitometry (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

Size-exclusion Chromatography

Syndecan-4 cytoplasmic tail peptides S4c or S4c-P (300 μM) were incubated with $PIP_2$ (350 μM, prepared as above) in 0.5 ml mM HEPES (pH 7.3), 150 mM NaCl, on ice for 30 min. Samples were applied at 4 °C to a Sephadex G-50 (Pharmacia Biotech, Uppsala, Sweden) 30 x 1.6 cm column equilibrated with the incubation buffer, and the absorbency of the flow through was measured at 280 nm.

Immunoprecipitation

Rat fat pad capillary endothelial cells (RFPEC, gift of Dr. R.D. Rosenberg, MIT (11) were grown to confluence in M199 medium supplemented with 10% FBS (Life Technologies) at 37 °C in a 5% $CO_2$ humidified atmosphere. The cells were harvested by trypsinization, lyzed, and subjected to immunoprecipitation with a cytoplasmic tail-specific antiserum as described previously in Experimental Series I.

Electrophoresis, Transfer, and Immunoblotting

Immunoprecipitated syndecan-4 cytoplasmic tail was re-suspended in Laemmli sample buffer and resolved by SDS-PAGE on a 4-20% Tris-glycine gel (BioRad), and transferred for 2 hrs at 250 mA in 150 mM glycine, 20 mM Tris-

HCl, and 20% methanol to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore). The membranes were immunoblotted as described previously in Experimental Series I using polyclonal antibodies to PKCα or to PKCδ (both at 2 μg/ml; purchased from Santa Cruz Biotechnology, Santa Cruz, CA).

Syndecan-4 Cytoplasmic Tail Peptide - PKC Binding Assay

Cytoplasmic tail peptides S4c or S4c-P (10 μM) were incubated on ice for 30 min either in the presence or absence of $PIP_2$ (20 μM; prepared as above) with recombinant PKCα (4 μM) in 0.5 ml of the same buffer used in the $PIP_2$ binding assay. The cytoplasmic tail peptide was immunoprecipitated, and the samples were resolved by SDS-PAGE, transferred, and immunoblotted as described above.

PKC In Vitro Assays

Samples (30 μl) consisted of PKCβ1 optimal substrate peptide (100 μM) either with or without syndecan-4 cytoplasmic tail peptides S4c or S4c-P (both at 50 μM) in 25 mM Tris-HCl (pH 7.4), 5 mL $MgCl_2$, 1 mM DTT, 50 μM ATP, and 5 μCi [γ-$^{32}$P]ATP (New England Nuclear, Boston, MA). In some assays the buffer was supplemented with either $PIP_2$ (50 μM), or PS (4 μg/ml), diolein (6.2 μg/ml), and 0.2 mM $CaCl_2$. In PKCδ assays the buffer was supplemented with PS and diolein as above, and with 0.5 mM EGTA. Upon addition of either PKCα (120 ng/ml) or PKCδ (430 ng/ml), samples were incubated at 30 °C for 10 min, and reactions were stopped by boiling in Laemmli sample buffer for 4 min. The samples were resolved on 16.5% Tris-tricine gels (BioRad Laboratories), transferred to PVDF membranes and detected as described previously in Experimental Series I.

**Experiment 6:**

Syndecan-4 cytoplasmic tail has been shown to activate a mixture of $Ca^{2+}$-dependent PKCs and of recombinant PKCα in the presence of $PIP_2$. To assess the effect of Ser$^{183}$ phosphorylation on syndecan-4-dependent PKC activation, the

ability of the 28 amino acid-long syndecan-4 cytoplasmic tail peptide, S4c, and S4c-P peptides to activate recombinant PKCα was studied using the PKCβ1 optimal substrate peptide in an in-vitro assay. The results are graphically illustrated by Fig. 5.

Fig. 5 shows the activation of PKCα cytoplasmic tail peptides. Densitometry histograms of autoradiographic images of PKC β1 optimal substrate peptide were resolved on 16.5% Tris-tricine gels and then transferred to PVDF membranes (n=3; bars denote SD). The substrate was phosphorylated in-vitro by recombinant PKCα (120 ng/ml) in the presence of PS (4 μl/ml), diolein (6.2 μg/ml), and 0.2 mM calcium (white bars), initially without co-factors (striped bars), and then in the presence of 50 μM $PIP_2$ (black bars). Assays were performed under each set of conditions in the absence (Cont), and in the presence of 50 μM non-phosphorylated (S4c), or phosphorylated (S4c-P) syndecan-4 cytoplasmic tail peptides. The inset shows autoradiographic images of PKC β1 optimal substrate peptide phosphorylated in the presence of 50 μM $PIP_2$.

When the assays were carried out with the standard cPKC cofactors PS, DAG and calcium, the presence of neither the S4c, nor the S4c-P peptides had any additional effect on the catalytic activity of PKCα, as shown by Fig. 5. The same result was observed in PKC assays where no cofactors were added. However, the assay conducted in the presence of $PIP_2$, together with the S4c peptide, revealed that the catalytic activity of PKCα towards the PKCβ1 peptide was approximately 10-fold larger than in assays with $PIP_2$ alone. On the other hand, when the S4c-P peptide was added instead of S4c, the phosphorylation level of the substrate was similar to that obtained with $PIP_2$ alone. Unlike PKCα, the S4c peptide did not activate PKCδ under the same conditions (data not shown). The activity of PKCα in the presence of the S4c peptide and $PIP_2$ was 72 ± 10% (±SD, n = 3) of its activity in the presence of the S4c peptide, PS, DAG, and calcium.

**Experiment 7:**

The ability of the unphosphorylated but not the phosphorylated cytoplasmic tail of syndecan-4 to activate PKCα in-vitro may relate to a reduced PKCα affinity upon phosphorylation of the cytoplasmic tail. Previous studies [Oh et al., J. Biol. Chem. 272: 8133-8136 (1997)] have demonstrated the ability of the cytoplasmic tail of syndecan-4 to bind PKC; and narrowed the identity of the bound PKC isozyme in-vivo down to a group of four (α, βI, βII, γ, and δ). Though PKCα was shown to bind to the cytoplasmic tail of syndecan-4 in-vitro, the cytoplasmic tail could also bind to and be a substrate of PKCδ. To determine the ability of syndecan-4 to bind PKCα or δ in-vivo, RFPEC lysates were immunoprecipitated with an antiserum specific to the cytoplasmic tail, and the immunoprecipitants were probed with antibodies specific either to the α or δ PKC isozymes. The results are shown by Figs. 6A-6C respectively.

Fig. 6 reveals the PKC binding to syndecan-4 cytoplasmic tail peptides. Fig. 6A shows immunoblots of recombinant PKCα (lane 1), and of syndecan-4 cytoplasmic tail immunoprecipitated from RFPEC lysate (lane 2). Samples were resolved on 4-20% Tris-glycine gels, transferred to PVDF membranes, and probed with a polyclonal antibody to PKCα. Fig. 6B is similar to Fig. 6A but lane 1 is an immunoblot of recombinant PKCδ, and the membrane was probed with a polyclonal antibody to PKCδ. Fig. 6C shows an immunoblot of recombinant PKCα (4 $\mu$M) incubated with 10 $\mu$M non-phosphorylated (lanes 1,2), or phosphorylated (lanes 3,4) syndecan-4 cytoplasmic tail peptides in the absence (lanes 1,3) or in the presence (lanes 2,4) of 20 $\mu$M $PIP_2$, and immunoprecipitated with antiserum specific to the cytoplasmic tail of syndecan-4.

As shown by Figs. 6A and 6B respectively, the presence of PKCα but not of PKCδ was detected in the immunoprecipitants. The results clearly evidence these facts.

To analyze the effects of syndecan-4 cytoplasmic tail phosphorylation on its ability to bind PKCα, in-vitro assays with recombinant PKCα and the S4c and S4c-P peptides were then performed. Incubation of PKCα with either peptide produced, however, similar degrees of binding both in the presence and absence of

$PIP_2$ (Fig. 6C). It follows, therefore, that PKCα binding is not affected by $Ser^{183}$ phosphorylation in the syndecan-4 cytoplasmic tail and thus cannot explain the effect of syndecan-4 phosphorylation on the enzyme's activity.

**Experiment 8:**

Both the oligomerization and PKCα activation capacities of the cytoplasmic tail of syndecan-4 have been reported as depending on the presence of $PIP_2$ [Oh et al., J. Biol. Chem. 272: 11805-11811 (1997); Lee et al., J. Biol. Chem. 273: 13022-13029 (1998)]. It was of interest, therefore, to determine whether the phosphorylation of $Ser^{183}$ in the cytoplasmic tail of syndecan-4 affects the affinity of the tail to $PIP_2$. To this end, the in-vitro binding between $PIP_2$ micelles and S4c or S4c-P peptides was compared using a filtration assay. The filter retains the $PIP_2$ micelle-bound peptide, while the unbound peptide passes through it. The results are illustrated by Figs. 7A and 7B respectively.

Fig. 7 shows the binding between $PIP_2$ and syndecan-4 cytoplasmic tail peptides. Fig. 7A shows the filter flow-through samples of non-phosphorylated (S4c) and phosphorylated (S4c-P) syndecan-4 cytoplasmic tail peptides, after incubation with $PIP_2$ at the indicated concentrations. Samples were resolved on 16.5% Tris-tricine gels. Fig. 7B graphically illustrates the densitometry results of the gel bands of the S4c (black bars) and S4c-P (white bars) peptides shown in Fig. 7A.

Fig. 7 demonstrates that the binding affinity of the S4c peptide to $PIP_2$, as determined by band densitometry of the SDS-PAGE-resolved filter flow-through samples, was significantly higher than that of the S4c-P peptide. At a peptide:$PIP_2$ molar ratio of 2:1, 50% of the S4c peptide that passed through the filter in the absence of $PIP_2$ was retained, versus none of the S4c-P peptide. Practically all the applied S4c peptide was retained by the filter at a peptide:$PIP_2$ molar ratio of 1:2, while as much as 50% of the S4c-P peptide still passed through the filter under the same conditions. The dissociation constants ($K_d$) calculated from the results shown

in Fig. 7 were 2.4 $\mu$M for the non-phosphorylated peptide (S4c), vs. 232 $\mu$M for the phosphorylated one (S4c-P). Thus, $Ser^{183}$ phosphorylation results in significant reduction in the ability of $PIP_2$ to bind to the cytoplasmic tail of syndecan-4.

**Experiment 9:**

Previous studies have demonstrated that the cytoplasmic tail of syndecan-4 undergoes oligomerization in the presence of $PIP_2$; furthermore this oligomerization appeared necessary for PKC$\alpha$ activation. The reduced affinity between the cytoplasmic tail and $PIP_2$ caused by phosphorylation could conceivably be accomplished by changes in the oligomerization properties of syndecan-4. To compare the oligomerization of the S4c peptide to that of the phosphorylated peptide S4c-P, both were incubated either in the presence or absence of $PIP_2$, as described in the Experimental Procedures, and passed through a size exclusion column. The results are graphically represented by Fig. 8A-8D respectively.

Fig. 8 shows the size-exclusion column chromatography of syndecan-4 cytoplasmic tail peptides. Figs. 8A and 8C present absorbency profiles at 280 nm of flow-through samples of non-phosphorylated samples; and Figs. 8B and 8D present phosphorylated syndecan-4 cytoplasmic tail peptides. The samples of Figs. 8A and 8B were incubated without $PIP_2$; while the samples of Figs. 8C and 8D contained $PIP_2$. The results shown in Figs. 8C and 8D are representative of two experiments.

As Figs 8A and 8B reveal, both peptides eluted as a single peak when incubated in the absence of $PIP_2$. When the mixtures are incubated in the presence of $PIP_2$, however, the S4c peptide eluted as two peaks - one of an approximate molecular mass of 7 kDa (Fig. 8C), and another heavier peak of a molecular mass greater than 17 kDa (the molecular mass of the heaviest molecular mass standard used in this experiment). The S4c-P peptide, on the other hand, eluted as a single peak of the same approximate molecular mass as the first peak of the S4c peptide (Fig. 8D). These results indicate the cytoplasmic tail of syndecan-4 loses its capacity to form oligomers upon phosphorylation of $Ser^{183}$. Based on the position of the first peaks of the S4c and the S4c-P peptides, it appears that both the S4c

and the S4c-P peptides formed dimers, similar to the behavior observed in the $PIP_2$-binding experiment (see Fig. 7). The broader peaks observed with both peptides when incubated in the presence of $PIP_2$, compared with the sharper ones obtained in the absence of $PIP_2$, reflect a wider spread in molecular weight, probably resulting from the range of $PIP_2$ binding to the peptides.

**Conclusions:**

1. This experiment series presents three distinct findings concerning the role of the syndecan-4 core protein in signal transduction: (a) phosphorylation of a single serine residue ($Ser^{183}$) located in the membrane-proximal part of the cytoplasmic tail of syndecan-4 reduces the affinity of the tail to the phosphoinositide $PIP_2$. Upon phosphorylation, the cytoplasmic tail loses its capacity to (b) undergo multimerization and to (c) activate PKCα in the presence of $PIP_2$. These findings provide the first evidence for a functional role of the phosphorylation of $Ser^{183}$ in the cytoplasmic tail of syndecan-4.

2. The capacities of the cytoplasmic tail of syndecan-4 to undergo multimerization and to activate PKCα were manifest only in the presence of $PIP_2$. The mechanism of PKCα activation by the cytoplasmic tail of syndecan-4 requires formation of cytoplasmic tail multimers. $Ser^{183}$ phosphorylation prevents this oligomerization by inhibiting $PIP_2$ binding to the variable region of the syndecan-4 cytoplasmic tail. It follows, therefore, that the loss of PKCα activation by the cytoplasmic tail upon phosphorylation of $Ser^{183}$ is a direct consequence of the concomitant reduction in affinity to $PIP_2$ and impaired multimerization. Since the cytoplasmic tail of syndecan-4 did not activate PKCδ, this activation may be specific to PKCα. On the other hand, $Ser^{183}$ phosphorylation had no effect on the capacity of the syndecan-4 cytoplasmic tail to bind PKCα. The ability of syndecan-4 to activate PKCα signaling in endothelial cells, the regulation of this signaling by syndecan-4 phosphorylation, and the previously demonstrated bFGF-dependent regulation of the state of syndecan-4 cytoplasmic tail phosphorylation reveals the existence of a novel bFGF-dependent signaling pathway.

The present invention is not restricted in form nor limited in scope except by the claims appended hereto.

What we claim is:

1. A method for stimulating angiogenesis within variable cells, tissues, and organs in-situ, said method comprising:

identifying a viable endothelial cell in-situ as a target, said targeted endothelial cell bearing a plurality of transmembrane syndecan-4 proteoglycans positioned at and through the cell surface wherein the 183rd amino acid residue present within the intracellular cytoplasmic domain of said syndecan-4 proteoglycan is a serine residue;

administering to said targeted endothelial cell on at least one occasion a predetermined amount of an inhibitor of Protein Kinase C δ (delta) isoenzyme activity such that said 183rd serine residue within the cytoplasmic domain of at least some of said syndecan-4 proteoglycans is present in an non-phosphorylated state; and

allowing said 183rd serine residue within the cytoplasmic domain of said syndecan-4 proteoglycans to continue to be present in a non-phosphorylated state, whereby a stimulation of angiogenesis in-situ results.

2. A method for stimulating angiogenesis within viable cells, tissues, and organs in-situ, said alternative method comprising:

identifying a viable endothelial cell in-situ as a target, said targeted endothelial cell bearing a plurality of transmembrane syndecan-4 proteoglycans positioned at and through the cell surface wherein the 183rd amino acid residue present within the intracellular cytoplasmic domain of said syndecan-4 proteoglycan is a serine residue;

administering to said targeted endothelial cell on at least one occasion a predetermined amount of a composition able to increase Protein Kinase C α (alpha) isoenzyme activity intracellularly such that said 183rd serine residue within the cytoplasmic domain of at least some of said syndecan-4 proteoglycans is present in an non-phosphorylated state in-situ; and

allowing said 183rd serine residue within the cytoplasmic domain of said syndecan-4 proteoglycans to continue to be present in a non-phosphorylated state, whereby a stimulation of angiogenesis in-situ results.

3. A method for stimulating angiogenesis within viable cells, tissues, and organs in-situ, said alternative method comprising:

identifying a viable endothelial cell in-situ as a target, said targeted endothelial cell bearing a plurality of transmembrane syndecan-4 proteoglycans positioned at and through the cell surface wherein the 183rd amino acid residue present within the intracellular cytoplasmic domain of said syndecan-4 proteoglycan is a serine residue;

administering to said targeted endothelial cell on at least one occasion a predetermined amount of an substance able to activate at least one enzyme selected from the group consisting of protein phosphatases 1 and 2A such that said 183rd serine residue within the cytoplasmic domain of at least some of said syndecan-4 proteoglycans is present in an non-phosphorylated state; and

allowing said 183rd serine residue within the cytoplasmic domain of said syndecan-4 proteoglycans to continue to be present in a non-phosphorylated state, whereby a stimulation of angiogenesis in-situ results.

4. The method as recited in claim 1 wherein said inhibitor of Protein Kinase C δ (delta) isoenzyme activity is selected from the group consisting of chemical PKC inhibitors, over-expressed DNA encoding the pseudosubstrate domain of PKC δ (delta) isoenzyme, and prepared anti-sense PKC δ (delta) oligonucleotide sequences.

5. The method as recited in claim 2 wherein said composition able to increase Protein Kinase C α (alpha) isoenzyme activity is selected from the group consisting of phorbol esters, over-expressed DNA encoding the PKC α (alpha) isoenzyme molecule, and over-expressed DNA encoding the PKC α (alpha) catalytic domain.

6. The method as recited in claim 3 wherein said activating substance is selected from the group consisting of over-expressed DNA encoding the catalytic subunits of the protein phosphatase and over-expressed DNA encoding the regulatory units of the protein phosphatase.

Fig. 1

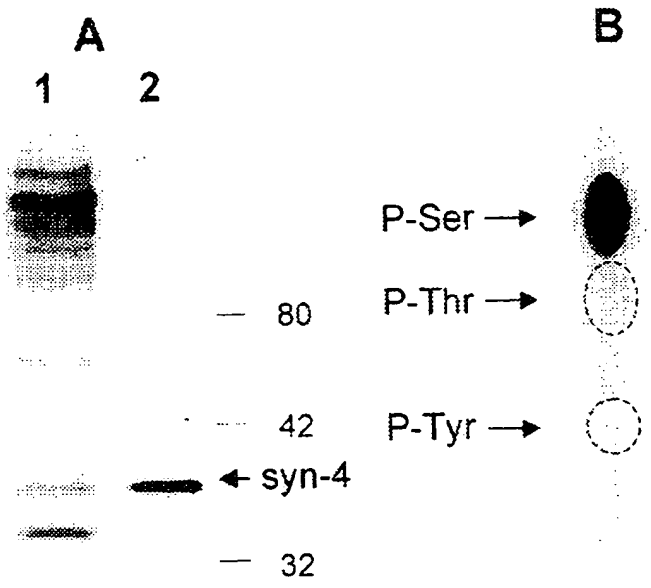


Fig. 2

Fig. 3

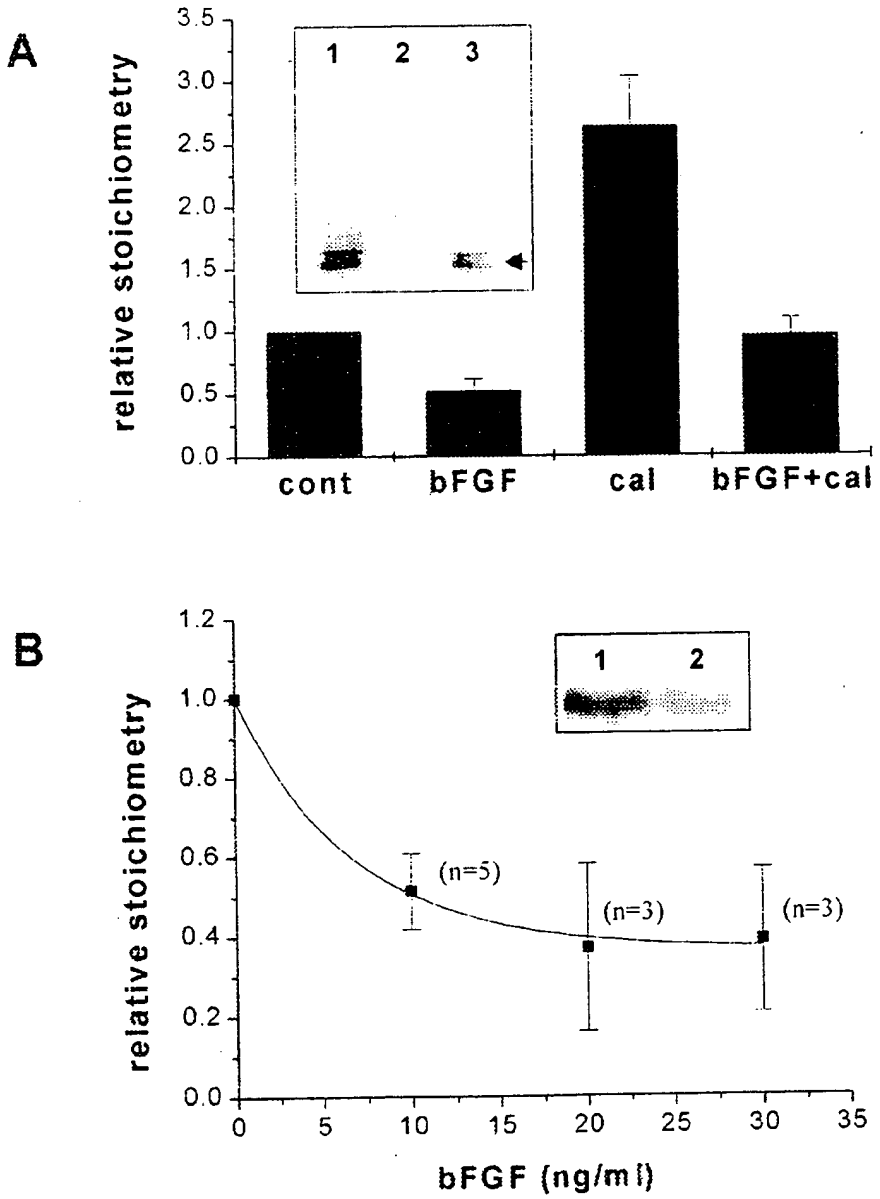

A
relative stoichiometry
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3
cont
bFGF
cal
bFGF+cal
B
relative stoichiometry
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 2
(n=5)
(n=3)
(n=3)
0 5 10 15 20 25 30 35
bFGF (ng/ml)

Fig. 4

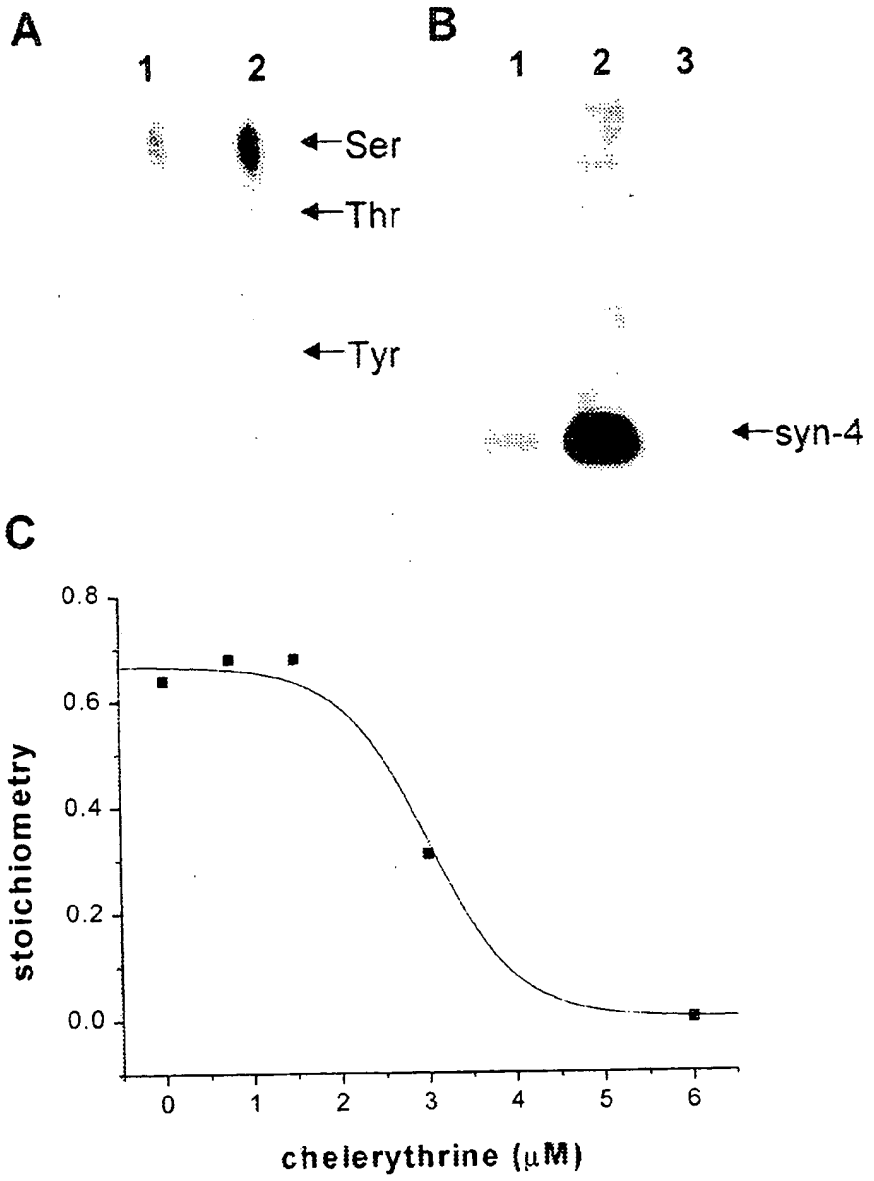

A
1
2
Ser
Thr
Tyr
B
1
2
3
syn-4
C
stoichiometry
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
chelerythrine (μM)

Fig. 5

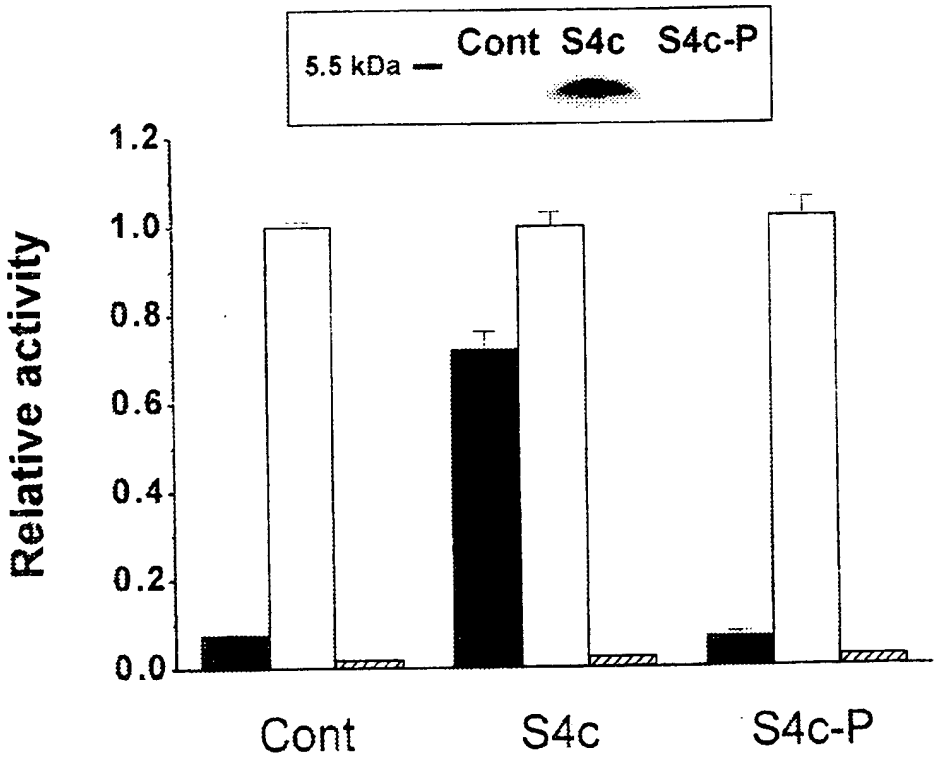

5.5 kDa
Cont S4c S4c-P
Relative activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cont
S4c
S4c-P

Fig. 6

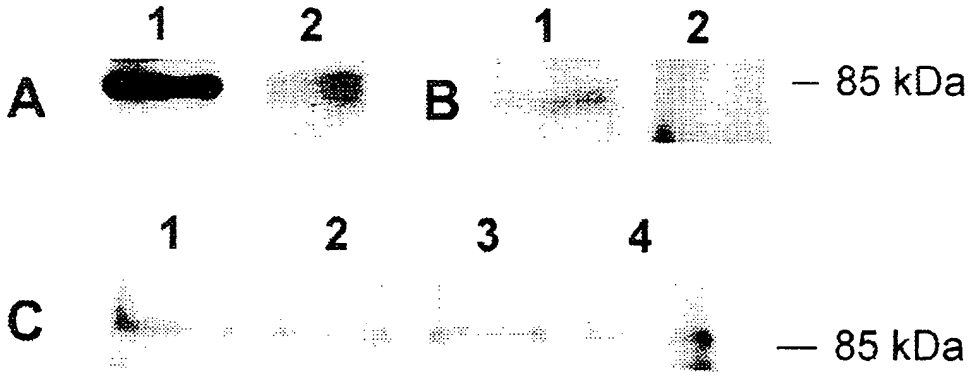


Fig. 7

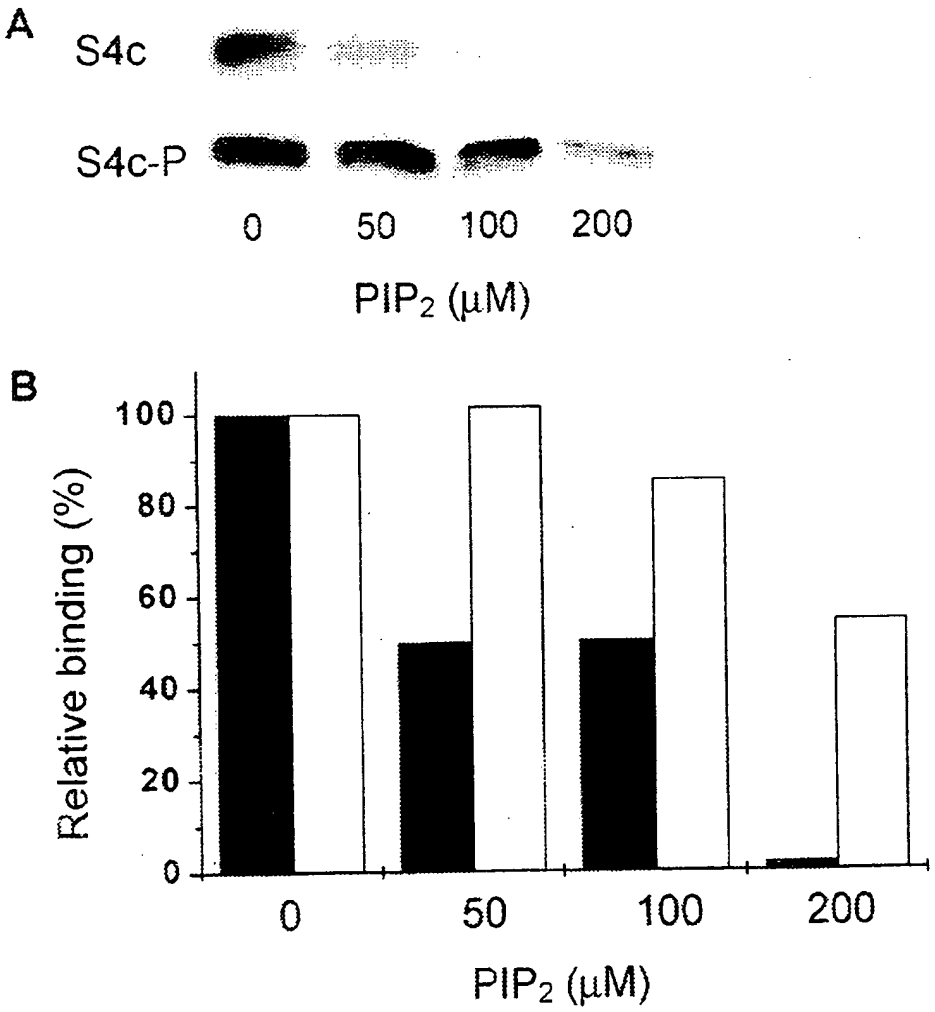
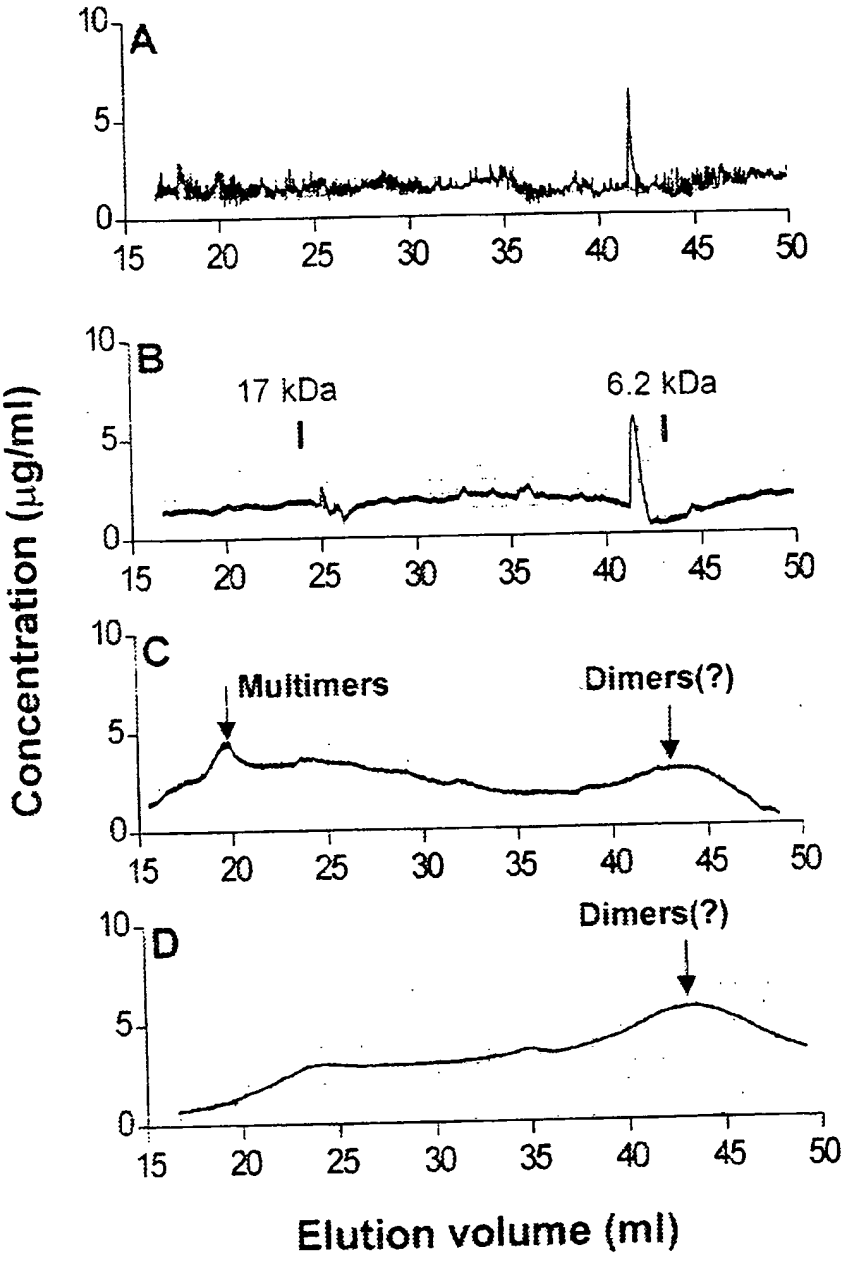

A
S4c
S4c-P
0
50
100
200
PIP2 (μM)
B
Relative binding (%)
100
80
60
40
20
0
0
50
100
200
PIP2 (μM)

Fig. 8


A
B
C
D
17 kDa
6.2 kDa
Multimers
Dimers(?)
Dimers(?)
Concentration (µg/ml)
Elution volume (ml)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26647

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :Please See Extra Sheet.
US CL :435/455; 514/2, 44; 536/23.5, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455; 514/2, 44; 536/23.5, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | GALLO et al. Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. Proc. Natl. Acad. Sci. USA. November 1994. Vol. 91, pages 11035-11039, entire document. | 1-6 |
| A | KOJIMA et al. Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. J. Biol. Chem. 05 March 1992. Vol. 267, No. 7, pages 4870-4877, entire document. | 1-6 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>24 FEBRUARY 2000 | Date of mailing of the international search report<br>05 APR 2000 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>ANNE-MARIE BAKER, Ph.D.<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26647

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | LEE et al. Solution structure of a syndecan-4 cytoplasmic domain and its interaction with phosphatidylinositol 4,5-bisphosphate. J. Biol. Chem. 22 May 1998. Vol. 273, No. 21, pages 13022-13029, entire document. | 1-6 |
| A | NISHIKAWA et al. Determination of the specific substrate sequence motifs of protein kinase C isoenzymes. J. Biol. Chem. 10 January 1997. Vol. 272, No. 2, pages 952-960, entire document. | 1-6 |
| A | OH et al. Multimerization of the cytoplasmic domain of syndecan-4 is required for its ability to activate protein kinase C. J. Biol. Chem. 02 May 1997. Vol. 272, No. 18, pages 11805-11811, entire document. | 1-6 |
| A | OH et al. Syndecan-4 proteoglycan regulates the distribution and activity of protein kinase C. J. Biol. Chem. 28 March 1997. Vol. 272, No. 13, pages 8133-8136, entire document. | 1-6 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26647

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 38/00, 48/00; C07H 21/04; C12N 15/63

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST (all databases)
Dialog (file: medicine)
search terms: m. simons, a. horowitz, syndecan 4, phosphorylat?, angiogenesis, serine, protein kinase C, PKC, protein phosphatase 1, protein phosphatase 2A, inhibit?